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OF THE

Woods Hole, Mass.

Marine Biological Laboratory

WOODS HOLE, MASS.

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BIOLOGICAL BULLETIN

RESPIRATORY METABOLISM.

PHYSIOLOGICAL STUDIES ON RESPIRATORY METABOLISM.[1]

JOSEPH HALL BODINE AND PAUL RUDBERT ORR.

ZOÖLOGICAL LABORATORY, UNIVERSITY OF PENNSYLVANIA.

INTRODUCTION.

Such quantitative knowledge as now exists concerning the fruit fly, *Drosophila,* has been largely obtained from genetical studies by Morgan and his students (1). Quantitative physiological studies on this organism, however, have been carried out principally by Loeb and Northrup (2) and more recently by Pearl and his associates (3). These authors have been concerned primarily with studies on the duration of life of the organism under normal as well as experimental conditions. Inasmuch as no quantitative information seems to exist concerning such questions as the physiological differences between stocks or strains, the physiological differences between sexes, etc., it was deemed advisable to study these questions in some detail. The present paper is one of a series dealing with certain physiological phenomena such as, first, changes in the oxygen and carbon dioxide content of culture bottles; secondly, the rates of carbon dioxide output; thirdly, the rates of oxygen consumption of two different stocks or strains of animals, the so-called ' wild ' and ' vestigial ' types.

MATERIAL AND METHODS.[2]

Two different stocks or strains of *Drosophila melanogaster,* the so-called " wild " and " vestigial " types, were used in these ex-

[1] Hereditary differences in respiratory metabolism of two stocks of *Drosophila.*

[2] Parts of the data in this paper are taken from a thesis submitted by P. R. O. to the graduate school of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Master of Science.

periments. These stocks were obtained from Dr. R. L. King of this laboratory and have been bred continuously by him for many generations. The vestigial stock is a synthetic one carrying two second chromosome mutations, vestigial and black, in homozygous form.

The stocks were carried along in the laboratory at room temperatures in quart milk bottles and fed the usual banana-agar-

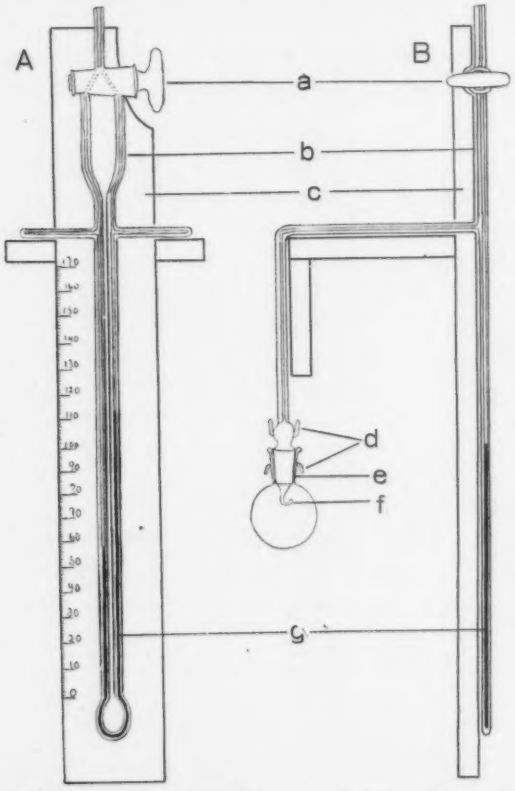

FIG. 1. Diagram of modified Krogh micro-respiration apparatus. *A*, front view. *B*, side view. *a*, glass-ground stopcock; *b*, capillary tubing with bore of approximately 0.4 mm. in diameter; *c*, wooden stand for supporting manometer; *d*, glass hooks for holding respiration chamber to manometer by means of elastic bands; *e*, glass-ground joint; *f*, glass spoon support for organism; *g*, kerosene in manometer.

Note that entire manometer is made of glass with ground connections.

yeast mixture as used by geneticists and others working with flies (Pearl 3). The experimental animals were kept in 100 cc. culture bottles in an electric incubator at 25° C. Small square pieces of filter paper were put in the culture bottles so that the larvæ could crawl up on them and pupate. The filter paper with the attached pupæ were removed at regular intervals and pupæ of known age thus obtained. The pupæ were carefully removed from the filter paper, cleaned and washed, then sterilized in 95 per cent. alcohol and again washed. They were then put into

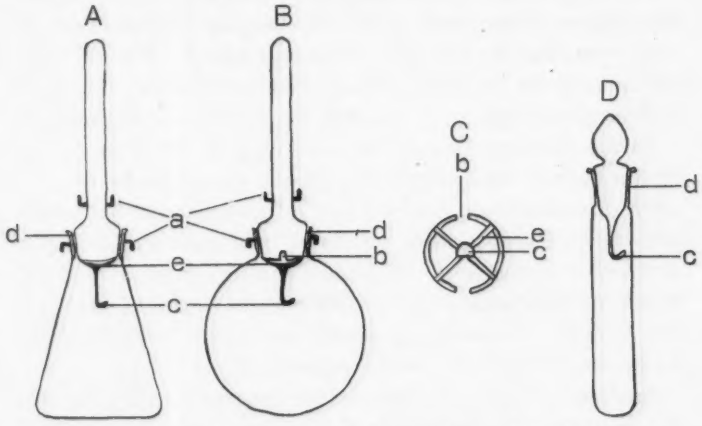

FIG. 2. Diagrams of different types of apparatus used in CO_2 determinations. *A*, flask of approximately 250 cc. capacity, which can be used either as a standard or as a respiration chamber. *B*, same as *A*. *C* shows attachment of animal holder (*e*) as well as trough (*b*). *D*, respiration chamber, 3 cc. capacity. *a*, glass hooks for holding parts of flask together by means of elastic bands; *b*, trough in glass to allow indicator solution to run from flask to attached comparing tube without coming in contact with supports of animal holder or spoon; *c*, glass animal holder or spoon; *d*, glass-ground joints; *e*, glass supports for animal holder or spoon. Apparatus is made entirely of Pyrex or Non-sol glass with no rubber stoppers or connections. Joints are all ground.

small shell vials containing small pieces of moistened filter paper to prevent drying. The vials were marked, stoppered with a small cotton plug and kept in the incubator at 25° C. Great care is necessary in handling individual pupæ because of their small size and the fact that they are easily injured. A very thin metal spat-

ula has been found a most convenient instrument for handling the organisms. In weighing pupæ a delicate analytical balance was used.

Since the purpose of the present investigation was primarily to determine the rates of oxygen consumption and carbon dioxide output of the different stocks of flies, it was necessary to have methods accurate and delicate enough to measure the respiratory exchange of such small organisms. The following types of apparatus were found to fulfill these requirements.

For oxygen determinations a modified Krogh (4) micro-respiration apparatus was used. The accompanying diagrams and explanations show the apparatus as finally adopted. For the methods of using the apparatus, derivation of formulæ, etc., the reader is referred to Krogh's (4) complete descriptions and explanations.

Carbon dioxide determinations were made by the Haas (5) indicator method, using suggestions pointed out by Jacobs (6). A decided improvement over the method as suggested by Jacobs, however, is apparatus made entirely of hard glass with no rubber stoppers or connections. The accompanying diagrams and explanations show some of the various types of apparatus employed. For methods of calculating results, etc., with this method the reader is referred to the article by Jacobs (6).

Analyses of the air in culture bottles were made with a Haldane gas apparatus (7), the sample of air being slowly drawn out of the bottle into the apparatus by means of a capillary tube.

Analysis of Air in Culture Bottles.

Pearl and Parker (3) in recent studies on duration of life in Drosophila have pointed out that in the case of wild type, " . . . an increase of roughly 10 per cent. in the mean duration of life is brought about by increasing the ventilation of culture bottles, by covering the mouth with one layer of number 48 mesh bolting cloth as compared with the use of cotton plug stoppers as is the usual practice in cultures of *Drosophila* in the laboratory." Similar experiments were also carried out by these authors using Quintiple type flies and no such differences in duration of life were noted. Inasmuch as these authors report no analyses of the air contained in culture bottles with such stoppers it was thought

TABLE I.

SHOWS AMOUNTS OF CO_2 AND O_2 IN CULTURE BOTTLES STOPPERED WITH VARIOUS TYPES OF STOPPERS FOR DIFFERENT TIME INTERVALS AND ALSO AMOUNTS OF CO_2 AND O_2 IN ROOM IN WHICH BOTTLES WERE KEPT.

Capacity of Bottles—cc.	Time of Analysis after Culture was Begun—Hrs.	Media Used.	Presence or Absence of Flies.	Kind of Stopper.	Analysis, %.			
					Bottle.		Room.	
					CO_2.	O_2.	CO_2.	O_2.
110	24	Normal	+	Cotton plug	0.0114	20.8	0.014	20.86
110	24	"	+	" "	0.018	20.8	0.014	20.86
110	168	"	+	" "	0.05	21.08	0.018	20.5
110	236	"	+	" "	0.052	20.3	0.0103	20.3
110	24	"	+	Gauze plug	0.015	19.6	0.014	20.86
110	24	"	+	" "	0.0154	20.5	0.014	20.86
110	24	"	+	" "	0.0152	20.5	0.014	20.86
110	192	"	+	Gauze stretched over top	0.0518	21.59	0.016	20.5
110	360	"	+	" " " "	0.053	20.4	0.0103	20.3
110	192	"	+	Wire gauze stretched over top	0.0426	20.9	0.016	20.5
110	192	"	+	" " " " "	0.037	21.2	0.016	20.5
110	360	"	+	" " " " "	0.0309	20.1	0.0107	20.3
110	168	"	+	Gauze stretched over top	0.058	21.8	0.018	20.5
110	192	"	+	" " " "	0.0375	20.3	0.016	20.5
110	192	"	+	" " " "	0.032	21.8	0.016	20.5
75	120	"	+	Glass	61.2	5.3	0.018	20.5
75	120	"	+	"	53.09	5.35	0.018	20.5
110	192	"	+	Cork	41.9	9.03	0.018	20.4
110	288	"	+	"	55.4	4.1	0.018	20.4
75	288	None	+	Glass	2.17	16.2	0.018	20.4
110	288	Wet filter paper	+	Gauze stretched over top	0.015	21.03	0.018	20.4
75	282	" " "	+	Glass	3.9	16.3	0.018	20.4
110	48	Yeast and banana	−	Gauze stretched over top	0.016	21.6	0.018	20.4
110	48	" " "	−	Cotton plug	0.026	21.2	0.018	20.4
75	24	" " "	−	Glass	1.06	18.15	0.016	20.5
75	168	" " "	−	"	Gas formed; blew stoppers out.			

desirable to a least make accurate quantitative analysis of the carbon dioxide and oxygen content of the culture bottles used in the present experiments.

Culture bottles were variously stoppered as indicated in Table I. and analyses made of their carbon dioxide and oxygen contents. In making the analysis the air samples were at first taken from different levels in the bottles beginning just below the stopper and finally just above the medium. No marked differences in the carbon dioxide and oxygen content of the air samples from different levels in the bottles were noted except that when the sample was taken at the surface of the medium slight differences in amounts of the two gases were usually found. All samples used and indicated in Table I. were taken from the middle of the culture bottles.

An examination of this table shows that in bottles with cotton plugs, gauze plugs, gauze stretched over the tops, etc., there were no appreciable differences in the amounts of carbon dioxide and oxygen noted. In bottles stoppered with cork or glass rather marked increases in the carbon dioxide content and decreases in the oxygen content were found. It must be pointed out here, however, that in these experiments only carbon dioxide and oxygen have been measured and that no account of other gases formed has been taken into consideration.

Behavior of Flies in Differently Stoppered Bottles.

The behavior of flies kept in differently stoppered bottles as noted by Pearl and Parker (3) is of considerable interest. These authors found that in bottles stoppered with gauze the wild flies tended to congregate at the top of the bottle under the bolting cloth while no such behavior was noted in the case of the Quintiple flies. This behavior in the case of the wild flies was not noted in bottles stoppered with cotton. The increased amounts of fresh air in the case of the bottles stoppered with gauze is given by these authors as the cause of this behavior.

Experiments were carried out by the present authors to test the behavior of wild flies in differently stoppered culture bottles (capacity 100 cc.) but no significant differences were noted As a matter of fact if a cylinder was made of wire gauze the same size

as the culture bottles, and the two ends covered with white cloth gauze, the flies tended to congregate under the gauze cover when cylinder was placed in an upright position despite the fact that the composition of the air in the cylinder was uniform throughout. By changing the position of the culture bottles, as well as of the wire gauze cylinder, in respect to the source of light the flies could be made to congregate at either end whether cotton plugs, gauze, etc., were used as stoppers. It seems to the authors that factors other than ventilation, doubtless light, are responsible for such behavior of the flies in differently stoppered bottles.

Respiratory Metabolism.

Since the genetic constitution of wild and vestigial stocks differs greatly, it was deemed desirable to measure the rates of respiratory metabolism of the two types to see if in this respect equally marked differences could be detected. Such a comparison of respiratory metabolism requires that all body movements, etc., be eliminated and that the organisms be under identical environmental and physiological conditions throughout the experiments. To meet these requirements, in the present investigations, sterilized pupæ, kept under identical conditions, have been used. Since rates of respiratory metabolism are usually expressed per gram body weight per unit of time, it was also necessary to weigh the organisms used in the experiments. The general methods used in obtaining pupal weights were as follows: Pupæ of known age were carefully removed from the filter paper by means of a thin metal spatula, cleaned and washed, then sterilized in 95 per cent alcohol and again washed in water. The excess moisture was next removed by placing the pupæ on dry filter paper. They were then weighed on a delicate analytical balance, in lots of five and ten and the average weight per pupa determined.

Figure 3 based on the weights of several hundred individuals shows graphically the average body weights for pupæ of the two stocks at different ages. It is evident from an examination of this figure that vestigial pupæ are somewhat heavier than wild and remain so throughout their entire pupal life. Vestigials, however, do not produce as many pupæ as the wild. It is necessary, therefore, in experiments to have larger numbers of vestigial cul-

tures for the production of a certain number of pupæ on a particular day than in the case of the wild where the same culture can usually be used for several days. One gets a much larger number of body weights for one day old vestigial pupæ of different cul-

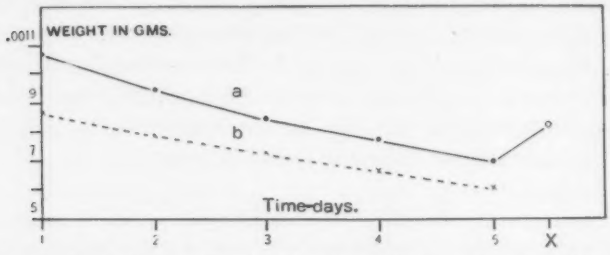

FIG. 3. Shows body weight of wild and vestigial pupæ from day of formation to emergence. Ordinate represents body weight per pupa in grams, abscissa, time in days indicated. X = fly just after emerging; a = vestigial; b = wild.

tures than for the wild pupæ where larger numbers of body weights for one day old pupæ are obtained from the same culture. In calculating average body weights, therefore, this fact plays an important part and should be kept in mind especially because of its significance in the determining of the rates of carbon dioxide output and oxygen intake per body weights of the animals. To control adequately such a factor it would doubtless be necessary to change the flies to new culture bottles every day so that the pupæ from the eggs laid in any one day could be obtained. If this factor is taken into consideration and a more extensive study made, we feel certain that a much smaller difference in the average body weights of the two stocks would be noted. It is also of interest to note from Fig. 3 that during the life of the pupæ there is steady decrease in weight. At the emergence of the fly, however, a rather marked increase in weight seems to occur and as a matter of fact the fly just after emerging weighs somewhat more than the pupa from which it came. This increased weight is doubtless due to absorption of water.

OXYGEN CONSUMPTION.

Inasmuch as the present paper deals only with a comparison of respiratory metabolism in the two stocks of flies, the data pre-

sented here are those obtained by using lots of five and ten pupæ at one time and making determinations daily throughout pupal life. The pupæ in all cases had been under identical environmental conditions and were of the same age. In running through any two series, *i.e.*, wild and vestigial, from the first day to the time of emergence, the same manometer was used for each, so that any possible error in the calibration of instruments would not enter into experiments dealing with the relative respiratory rates of the two stocks.

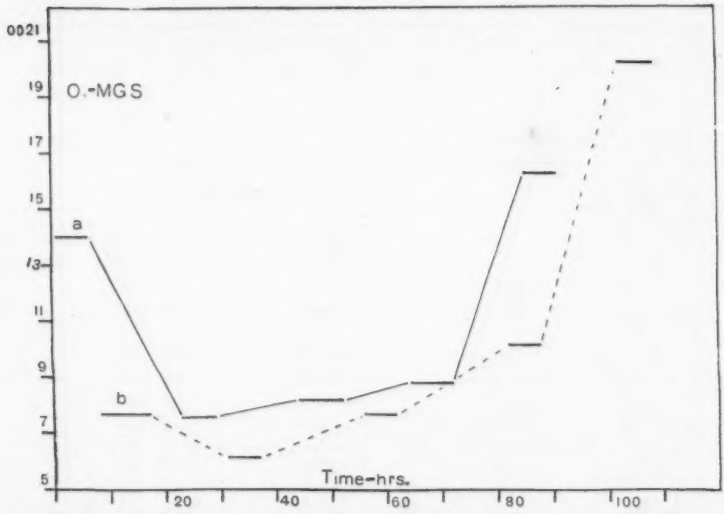

FIG. 4. Shows oxygen consumption (mg.) per mg. body weight per hour for wild and vestigial pupæ during entire pupal life. Ordinate represents oxygen (mg.) per mg. body weight per hour, abscissa time in hours indicated. *a* = wild; *b* = vestigial. Solid horizontal portions of curves indicate time during which oxygen determinations were made.

Table II. and Fig. 4, selected from many experiments, show the oxygen consumption for pupæ of the two stocks. From an examination of these it is evident that the rates of oxygen consumption for the two stocks are quite similar. In both stocks there is a decided decrease in oxygen consumption the second day followed by a gradual increase up to the time of hatching. The rate of oxygen consumption the fifth day usually exceeds that for the first day.

TABLE II.

SHOWS RATE OF O_2 CONSUMPTION FOR TWO LOTS OF TEN WILD AND TEN VESTIGIAL PUPÆ DURING ENTIRE PUPAL LIFE.

Temp. 22.5° C. Day of Pupal Life.	Oxygen (mm.³) per Min. per 10 Pupæ.		Oxygen (mg.) per Min. per Pupa.		Oxygen (mg.) per Mg. Body Weight per Min.	
	Wild.	Vestigial.	Wild.	Vestigial.	Wild.	Vestigial.
1.....	0.14170	0.09704	0.00002168	0.00001484	0.00002466	0.00001381
2.....	0.07097	0.06531	0.00001085	0.00000999	0.00001361	0.00001051
3.....	0.06807	0.07652	0.00001041	0.000011707	0.00001442	0.00001377
4.....	0.0690	0.09245	0.00001055	0.00001414	0.00001582	0.00001826
5.....	0.11780	0.16630	0.00001802	0.00002544	0.00002921	0.00003634

TABLE III.

SHOWS O_2 CONSUMPTION FOR FIVE DIFFERENT LOTS OF TEN WILD AND TEN VESTIGIAL PUPÆ DURING ENTIRE PUPAL LIFE.

Day of Pupal Life	Oxygen (mm.³) per Min. per Pupa	
	Wild	Vestigial
1	0.010146	0.008362
2	0.004531	0.007446
3	0.006699	0.011182
4	0.008459	0.014291
1	0.010020	0.010240
2	0.006434	0.007302
3	0.008194	0.008917
4	0.008892	0.016470
1	0.009909	0.008321
2	0.007426	0.008266
3	0.006790	0.011222
4	0.007846	0.011496
5	0.009470	0.013412
1	0.008200	0.015240
2	0.008449	0.007724
3	0.011587	0.011340
4	0.019070	0.017620
1	0.007405	0.009390
2	0.006730	0.006340
3	0.008660	0.010725
4	0.010870	0.011960

[3] It is a pleasure to acknowledge receipt of a grant from the Joseph Henry Fund of the National Academy of Science for the purchase of a sensitive water thermostat for this work.

Table III. shows results for five different lots of ten wild and ten vestigial throughout pupal life and also gives some idea of the range of variation existing in animals of the two types.

Carbon Dioxide Output.

Ten pupæ were used at one time and determination of carbon dioxide output made on successive days from the first day of pupation till hatching as in the case of oxygen determinations.

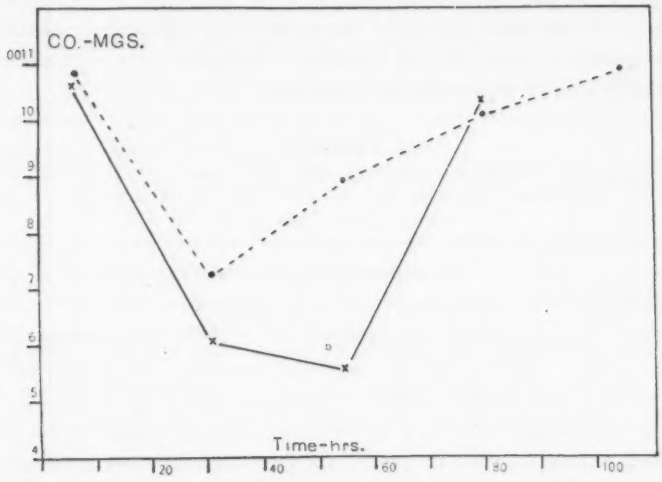

Fig. 5. Shows carbon dioxide output (mg.) per mg. body weight per hour for wild and vestigial pupæ during entire pupal life. Ordinate represents CO_2 (mg.) per mg. body weight per hour, abscissa time in hours indicated. a = vestigial; b = wild.

Table IV.

Shows Rate of CO_2 Production per mg. Body Weight per Minute for Two Lots of Ten Wild and Ten Vestigial Pupæ during Entire Pupal Life.

Day of Pupa Life.	CO_2 (mg.) per mg. Body Weight per min. Wild.	Vestigial.
1	0.00002704	0.00003052
2	0.00001025	0.00001230
3	0.00000934	0.00001493
4	0.00002197	0.00001840
5		0.00003321

1	0.00003129	0.00005140
2	0.00001462	0.00001928
3	0.00001465	0.00002017
4	0.00001583	0.00002759
5	0.00003942	0.00009972

Table IV. gives the amounts of carbon dioxide produced by ten wild and ten vestigial pupæ during their entire pupal life. Fig. 5 also shows graphically the rates of carbon dioxide production for two lots of pupæ. From an examination of this figure it will be noted that the curves follow closely those for oxygen consumption with characteristic decreases the second day, followed by steady increases up to the time of hatching. Vestigials seem to have the higher rate of carbon dioxide production.

TABLE V.

SHOWS TIME REQUIRED TO PRODUCE THE SAME AMOUNT OF CO_2 BY TEN DIFFERENT GROUPS OF TEN WILD AND TEN VESTIGIAL PUPÆ DURING ENTIRE PUPAL LIFE.

Day of Pupal Life.	Time to Produce Same Amount of CO_2—Minutes.			
	Wild.	Vestigial.	Wild.	Vestigial.
1	4	3	7	5
2	10	8	12	11
3	8	6	11	8
4	5	4	4	6
5	3	2		3
1	5	4	4	3
2	11	9	9	7
3	10	7	8	5
4	8	5	3	2
5	4	3		
1	3	2	6	4
2	10	6	7	8
3	8	4	7	6
4	2	1.5	3	2
1	4	3	6.5	5.75
2	8	7	10	8
3	7	6	9.5	7
4	6.5	4	2.3	5.3
5	2	1.5		2
1	5	3.5	1.5	1.3
2	8	9	6.7	5
3	8	8	4.8	4.5
4	3	7	2.8	4
5		2		2.3

Table V. gives examples of the variations in carbon dioxide production in ten different lots of ten wild and ten vestigial pupæ. A rather wide range of variation will be noted in the time taken by different lots of the same age to produce the same amount of carbon dioxide.

These studies are being continued in this laboratory, with the idea of investigating, in a quantitative manner, the possible effects of different factors upon the physiological make-up of the organism, as well as differences due to sex and species.

Summary and Conclusion.

1. No significant differences in air consumption (O_2 and CO_2) in culture bottles with different types of stoppers, all other conditions remaining the same, have been found.

2. There is a gradual decrease in body weight during pupal development of the fly.

3. Rates of oxygen consumption and carbon dioxide output of pupæ follow definitely shaped curves.

4. There is a decrease in oxygen consumption and carbon dioxide output the second day of pupal life followed by a gradual increase up to the time of hatching.

5. A comparison of the rates of respiratory metabolism of two types of flies, wild and vestigial, has been made and more extensive experiments seem necessary before differences found in these stocks can be definitely established.

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THE OCCURRENCE OF *RICKETTSIA*-LIKE MICROÖRGANISMS IN ADULT "LOCUSTS" (*TIBICEN SEPTENDECIM* LINN.).

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While attempting to extend some studies already made dealing with the distribution of *Rickettsia* in insect tissues[1] a number of "locusts" were collected in the vicinity of Teaticket, Massachusetts and were examined microscopically at the Marine Biological Laboratory. Eleven out of 50 of these specimens, or a little over 20 per cent., were found to be heavily infested with peculiar microorganisms which it is the purpose of this note to describe briefly.

In size they resembled *Rickettsia* in being somewhat smaller than most bacteria. After alcoholic fixation and coloration by Giemsa's method they measured from 0.2 to 0.3 micron in diameter and from 0.2 to 1.5 microns in length, with average dimensions of 0.2 by 1.0 micron. They were usually straight with bluntly rounded ends and were sometimes arranged in pairs (Figs. 1–3). Like *Rickettsia,* these microörganisms tended to be stained red by this technique; but unlike *Rickettsia,* they were somewhat resistant to decolorization by Gram's method—this was however a matter of degree in quality rather than one of distinct difference (Fig. 3). They were restricted in distribution to the lumina of the alimentary tract and Malpighian system—that is to say to two locations in which *Rickettsia* are very frequently met with. The manner in which they were seen to be applied in thick masses upon the epithelial surfaces was also suggestive of *Rickettsia* (Fig. 2). They were seldom observed within cells. In infested "locusts" the alimentary and Malpighian systems were not uniformly invaded throughout their whole extent. In serial sections it was a common experience to observe perfectly normal tubules, devoid of microörganisms, and then suddenly to encounter large numbers of them. In these areas the lining epithelium showed no

[1] Cowdry, E. V., *J. Exp. Med.*, 1923, XXXVII., 431.

noticeable modifications except a slight reduction in height. Although very careful search was made, none of the microörganisms were detected in the ovaries. Another point of similarity to *Rickettsia* was that the microörganisms did not reveal their presence by causing noticeable sickness in their insect-hosts.

Bacteria of larger size and staining blue by Giemsa's method were occasionally observed in small numbers within the body cavities of the same insects; but not in the intestinal lumina, or within the Malpighian tubules. Some of these bacteria are shown in the lower part of Fig. 2.

The alimentary tract, though well developed, was never distended with food but contained a small amount of a coagulum which might well have been produced by the action of enzymes and fixatives upon plant juices—accepting the evidence of Quaintance,[2] Snodgrass,[3] and others that the insects take food of this kind. Hargitt,[4] on the other hand, is of the opinion that the insects seldom if ever feed in the adult state. If substantiated, Hargitt's view will narrow down a search for the port of entry of the parasites.

The term "*Rickettsia*" has been applied to an ill-defined group of microörganisms which are difficult to classify with certainty owing chiefly to their resistance to ordinary methods of artificial cultivation. It was first used by da Rocha Lima,[5] in honor of Doctor H. T. Ricketts, who died while investigating typhus fever, to designate certain peculiar microörganisms which he found in coöperation with Doctor R. M. Wilder.[6] Since that time the word has gradually become generic in its scope and is now generally conceded to include also the parasite of Rocky Mountain spotted fever, certain organisms held to be responsible for trench fever and Japanese River sickness, as well as many which are not known to be pathogenic for man. About 25 different varieties of *Rickettsia* have thus far been reported. According to Wolbach,

[2] Quaintance, A. L., Bull. No. 87, Maryland Agr. Exp. Sta., 1902.

[3] Snodgrass, R. E., Smithsonian Report for 1919, published 1921, 381.

[4] Hargitt, C. W., Biol. Bull., 1923, XLV., 200.

[5] da Rocha Lima, H., *Berl. klin. Wchnschr.*, 1916, LIII., 567.

[6] Ricketts, H. T., and Wilder, R. M., *J. Am. Med. Assn.*, 1910, LIV., 1304, 1373.

Todd, and Palfrey:[7] "In every instance where careful study has been made it has been found—with the exception of the rickettsia of typhus—that the organisms pass down through successive generations in the eggs."

It is in connection with this possibility of hereditary transmission that further study of the microörganisms mentioned in this note may be interesting, particularly since their hosts exhibit a very unusual life history, some aspects of which have been made the subject of recent contributions to the BIOLOGICAL BULLETIN by Hargitt[4] and by Hickernell.[8] The particular Massachusetts brood, with which we are concerned, is possessed of a distinguished ancestry. Marlatt[9] quotes a description of the first swarm noted by settlers in 1634, in Moreton's words, as follows:

"It is to be observed that the Spring before this Sickness, there was a numerous company of *Flies*, which were like for bigness unto *Wasps* or *Bumble-Bees*, they came out of little holes in the ground, and did eat up the green things, and made such a constant yelling noise as made all the woods ring of them, and ready to deaf the hearers; they were not any of them heard or seen by the *English* in the country before this time: But the *Indians* told them that sickness would follow, and so it did . . . ."

By citing this historic reference and by mentioning certain points of resemblance between the microörganisms and *Rickettsia*, the implication is not intended that the "locusts," or their parasites, are in any way associated with human disease. During the great "locust" plague of 1868 and during every important reappearance of the insects before and since that time there have been many reports of the infliction of more or less serious stings. We have good authority (Riley) for assuming, however, that these stings are not caused by the "locusts" themselves but by other insects, such as digger wasps, which are often prevalent at the same time.

[7] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., "The Etiology and Pathology of Typhus." Harvard University Press. Cambridge, Mass., 1922, 222 pages.

[8] Hickernell, L. M., BIOL. BULL., 1923, XLV., 213.

[9] Marlatt, C. L., "The Periodical Cicada." Bull. No. 71, Bureau of Entomology, Washington, 1907, 181 pages.

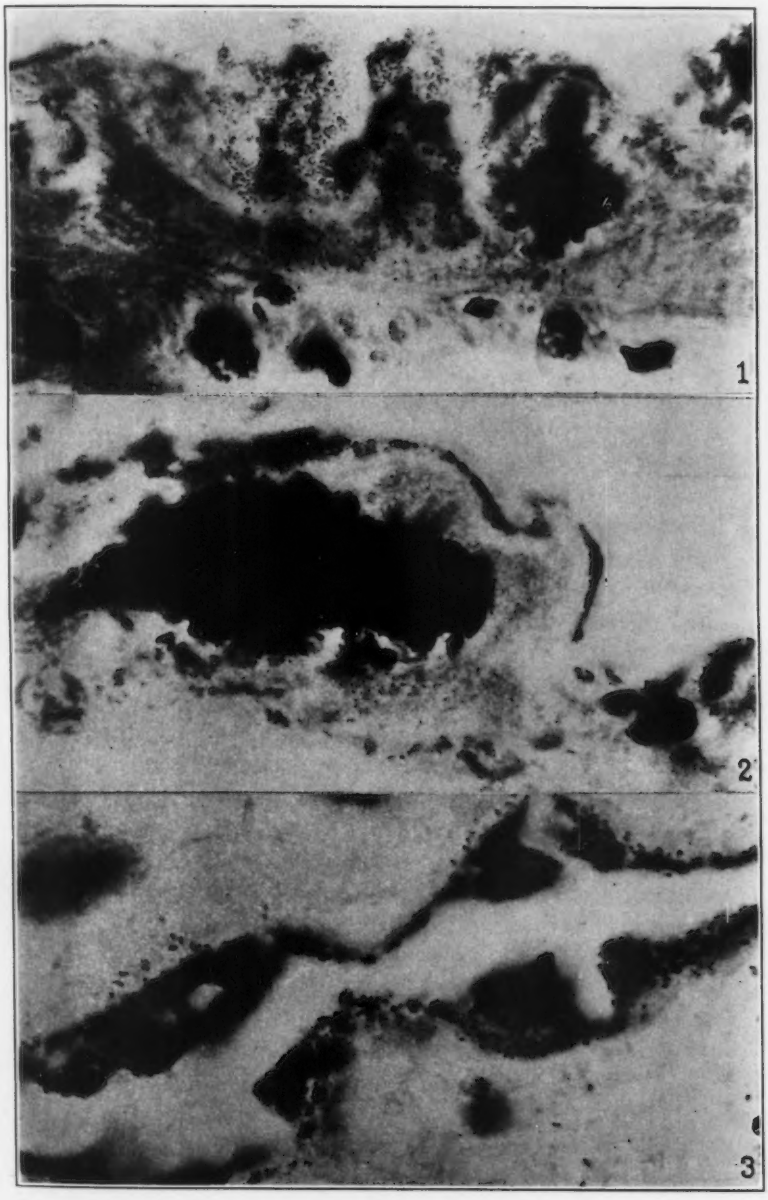
Returning to our Massachusetts brood, there is a gap in our records from 1634 to 1789 but published accounts [9] report the reappearance of the insects every 17 years from 1789 to 1923. There is every reason to believe that they have, with the utmost regularity, maintained this 17-year cycle for upwards of 300 years, with the prospect of continuing indefinitely, or until they are killed out by the removal of underbrush. Undoubtedly the *Rickettsia*-like microörganisms, to which attention has been called, have selected very remarkable hosts.

EXPLANATION OF PLATES.

FIG. 1. Intestine of adult "locust," alcoholic fixation, Giemsa's stain, and photograph at 1,000 diameters.

FIG. 2. Malpighian tubule of adult "locust," same fixation and staining, photograph at 1,000 diameters.

FIG. 3. Intestine of adult "locust," alcoholic fixation, Gram's stain, and photograph at 1,500 diameters.



E. V. COWDRY.

THE AXIAL GRADIENTS IN HYDROZOA.

VI. EMBRYONIC DEVELOPMENT OF HYDROIDS.

C. M. CHILD.

(From the Hull Zoölogical Laboratory, the University of Chicago.)

During the summers of 1917 to 1920, spent at the Puget Sound Biological Station, Friday Harbor, Washington, some time was spent in the study of polarity in eggs and embryos of several species of hydrozoa and of modification and control of development through differential susceptibility. Continuation, of the work has not been possible since 1920, but the data already at hand are consistent in themselves and constitute further evidence for the existence and importance in development of the physiological gradients as well as a basis for interpretation in physiological terms of the transformation of the planula into the hydroid form. This opportunity is taken to acknowledge again my obligations to the Director of the Puget Sound Biological Station for the facilities placed at my disposal.

Material and Methods.

Because of its abundance the hydromedusa, *Phialidium gregarium* constituted the chief source of embryonic material. Since the medusæ shed eggs and sperm more or less continuously in small quantities, fertilized eggs were most readily obtained in quantity by keeping medusæ in considerable numbers, often several hundred, in large pans or tubs for a few hours and then collecting eggs and embryos from the bottom. In such material stages may range from newly shed eggs to various cleavage stages or early blastulæ, according to the length of time the medusæ have been in the container. Embryonic stages of *Stomotoca atra* were obtained in the same way, but the medusæ of this species were usually much less abundant than *Phialidium*. Ovarian eggs of all stages of growth were obtained directly from the ovaries of these species. In a third species, *Gonothyræa clarkii*, which was used to some extent, the free swimming medusa stage is absent,

the early embryonic development occurring in gonophores on the hydroid. Planulæ of this species were obtained by keeping the gonophore-bearing hydroids in the laboratory for a few days and earlier stages, by opening the gonophores. The development of *Phialidium* and *Gonothyræa* was followed through the transformation of planula into hydroid stage, that of *Stomotoca* only to the late planula. *Phialidium* and *Gonothyræa* give rise to campanularian hydroids; *Stomotoca* is an anthomedusa.

The axial physiological gradients in eggs and developmental stages were demonstrated as susceptibility gradients by disintegration or cytolysis in KCN in indicated concentrations, *m*/100 to *m*/500 in sea water, $HgCl_2$ in indicated concentrations, *m*/50,000 to *m*/500,000 in sea water, methylene blue and neutral red in low concentrations in sea water; second, as gradients in rate and amount of reduction of $KMnO_4$ in various low concentrations in sea water and in rate of reduction of methylene blue; third, as gradients in rate of penetration of the vital dyes, neutral red and methylene blue. The concentrations of agents used are given as "indicated concentrations" because the normal medium sea water is used as solvent in all cases and the concentration given is merely that indicated by the amount of the agent which is added to a certain volume of sea water. As regards $KMnO_4$ and the vital dyes, the same results are obtained with a wide range of concentrations because these substances are taken up from very low concentrations and accumulate in unchanged or reduced form in the protoplasm. The point of chief importance is that the concentration be low enough so that the staining or disintegration by the dyes and the reduction of $KMnO_4$ shall not occur so rapidly as to obscure the differences at different levels.

A $KMnO_4$ solution, indicated concentration *m*/1,000, served as stock solution and was diluted two to five times or even more for use in demonstrating differences in rate of reduction. Frequently a drop or two of the stock solution was added from time to time as reduction decreased the concentration. Very low concentrations of $KMnO_4$ *e.g.*, below *m*/10,000, produce more or less cytolysis and can be used like other cytolytic agents to demonstrate the gradients. Special attention may be called to the fact that the permanganate gradient is not simply a gradient in the rate of re-

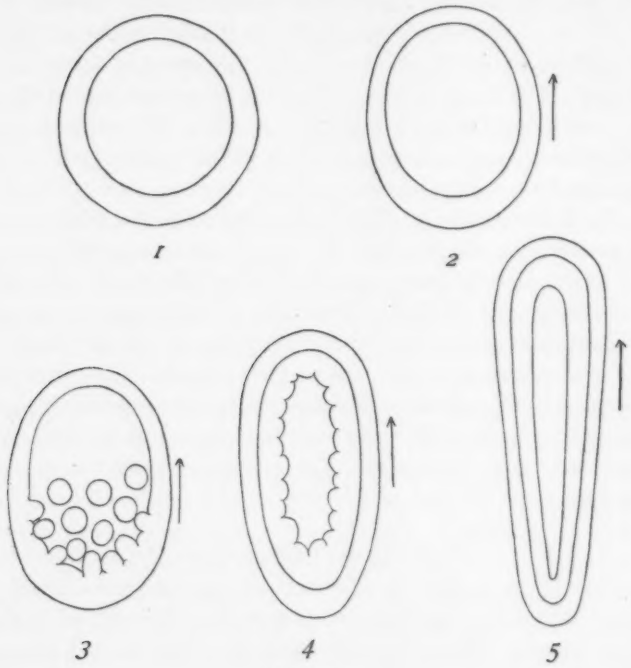
duction of $KMnO_4$ but when the reaction proceeds to completion in excess of $KMnO_4$ a gradient in depth of color indicating differences in amount of reduction is evident. For demonstration of these differences the material is kept in excess of $KMnO_4$ solution 24 to 48 hours and is then dehydrated and cleared since it is opaque in aqueous medium. Such cleared preparations are not permanent in any of the clearing agents thus far used nor in balsam. The brown or blackish color due to MnO_2 or other oxides gradually disappears, apparently by solution, in the course of several days longer. The preparations are sufficiently permanent, however, to permit imbedding and sectioning before the color gradient disappears, though of course fading occurs more rapidly in the sections than in the whole preparations.

For demonstration of the gradients by methylene blue various concentrations, ranging from 1/1,000 to 1/20,000 were used, according to species and result to be attained, the higher concentrations being used chiefly for differential staining, rapid death and disintegration, the lower for demonstration of the gradients by differential reduction and slow disintegration. Concentrations of neutral red used were all very low but were not determined because the results differ only as regards time with all concentrations used and because the dye gradually crystallizes out of solution.

The Polar Gradients During Normal Development.

The course of early development under normal conditions is very similar in the species used as material. Cleavage gives rise to a spherical ciliated blastula (Fig. 1) which soon begins to elongate and shows polarity in the graded thinning of the cell wall decreasing from apical to basal pole, and in locomotion with the apical end in advance (Fig. 2). Immigration of cells from the basal hemisphere, chiefly from regions near the basal pole, begins within a few hours (Fig. 3) and the cells which immigrate apparently undergo further division and arrange themselves as an inner entodermal layer as elongation of the larva continues (Fig. 4). The final form of the swimming planula, attained in *Phialidium* after about forty-eight hours at laboratory temperature, is approximately that shown in Fig. 5, but a considerable amount

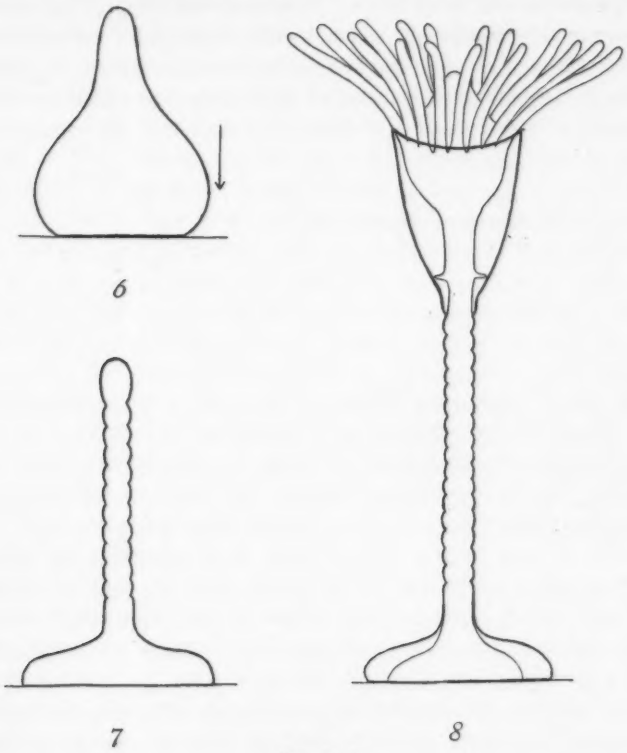
of slow contraction and extension occurs, the same planula being at times almost worm-like in form or contracting to a form considerably shorter and broader than Fig. 5. The original apical end still precedes in locomotion, as indicated by the arrows (Figs. 2–5). After a day or more of continued swimming, either free or along the bottom, the planula attaches itself by the original



FIGS. 1–5.

apical end, which shortens and thickens (Fig. 6), and the basal end begins to elongate to form the stem (Fig. 7) and this in turn gives rise at its tip to the first hydranth (Fig. 8). Stolons may arise later as outgrowths from the attached end. Stem and stolon are easily distinguished since the stem grows erect or free from the substratum and has an annulated perisarc, while the stolon grows in contact with the substratum and is without annulation. This course of development is essentially similar to that described by other investigators for various other hydroid species.

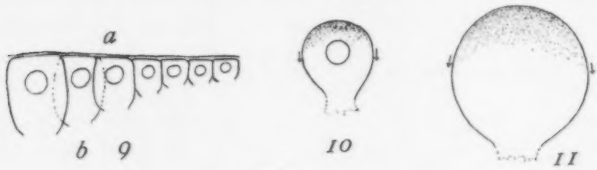
The evidence from the course of development itself, from differential cytolysis, from differential rate and amount of reduction of $KMnO_4$, from differential staining by neutral red and methylene blue and differential reduction of the latter, agrees in indica-



FIGS. 6–8.

ting, first the existence of a physiological gradient along the polar axis from the oöcyte to the planula stage with its high end at the apical end of egg and larva, and second, the appearance in the late planula of a second gradient at the basal end, opposite in direction to the first, and associated with the development of stem and hydranth from this end. Figs. 10 to 17, illustrating diagrammatically the polar gradients of various stages, are in part early stages of the reduction gradients with $KMnO_4$ (Figs. 10–12), in part

early stages of cytolytic gradients. Although complete series of data up to the first hydranth were obtained with cytolysis, with reduction and with differential staining, complete series of figures for the various methods are believed to be quite unnecessary since the results of all agree. The small arrows in Figs. 10–17 indicate the direction in which further progress of reduction or cytolysis occurs. As regards Figs. 10–12 it should also be noted that the shaded portions represent those regions in which the color resulting from reduction of $KMnO_4$ is deepest. All portions of



FIGS. 9–11.

the egg or later stage become diffuse yellow in permanganate within a few moments, but the differential staining of different regions becomes more and more distinct as reduction proceeds, until the increasing opacity obscures the differences and dehydration and clearing are necessary to make them visible.

The growing oöcytes of *Phialidium* form a more or less regular columnar epithelium in the gonad (Fig. 9), each cell being attached by its inner end (*b*) adjoining the radial canal, while the superficial free pole (*a*) is separated from the sea water only by a very delicate membrane covering the gonad. Except in very early oöcytes, the nucleus lies nearer the free pole. Growing oöcytes of various stages, isolated by teasing, rapidly assume rounded form and the nucleus often breaks down or is extruded, but the region of attachment remains distinguishable and serves as a landmark. Such isolated oöcytes show uniformly a gradient in susceptibility with all agents used, in reduction of permanganate and in differential staining, the cytolysis or change in aggregation of the cytoplasm, the reduction and the staining beginning at the free pole and progressing toward the attached pole, as indicated in Figs. 10 and 11. Nucleated and non-nucleated egg fragments also show a gradient which is doubtless identical with that of the

whole oöcyte, though the absence in most cases of any visible basis for orientation of the fragments makes complete demonstration of identity impossible. In the oöcyte of *Stomotoca* the gradient shows the same relation to free and attached poles of the cell as in *Phialidium*.

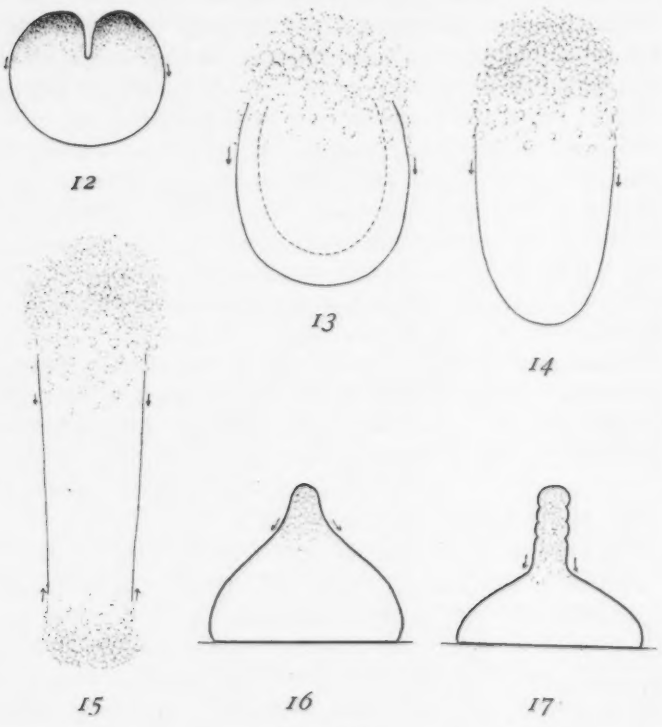
It has been repeatedly pointed out that the various lines of evidence concerning the gradients indicate very clearly that they are associated with quantitative differences in physiological condition involving metabolism as an important factor.[1] In the light of this evidence the inference is justified that the differentials in susceptibility, reduction and differential staining in these hydrozoan oöcytes are indicators of a quantitative physiological gradient in the cytoplasm. The relation of this gradient to the free and attached ends of the oöcyte in the gonad suggests that it is determined by the differential exposure of the cell. The intake of oxygen and elimination of Co_2 undoubtedly occur more readily at the free end than elsewhere, and there is every reason to believe that the persistence of such a differential during the growth period will establish a physiological gradient, primarily quantitative in character, involving metabolic and physical factors. In short, the facts at hand indicate, though they do not demonstrate that the physiological polarity of the oöcyte is determined by its differential exposure in the gonad.

The gradient persists after the egg is shed, the polar bodies are formed at its high end, *i.e.*, the pole of highest susceptibility and reducing power, and the first cleavage furrow cuts through the egg from this pole (Fig. 12). During cleavage and early blastula stages a gradient is continuously present, undoubtedly the same as that of the early stages, but absence of definite landmarks makes complete demonstration of identity impossible. As soon as directed locomotion and elongation of the blastula begin, we find that the gradient is apico-basal, the high end being at the apical pole of the blastula (Fig. 13) and the planula (Fig. 14). The facts leave little room for doubt that the gradient, apparently determined by differential exposure in the oöcyte, represents the polar axis of the egg and larva. Moreover, maturation, cleavage,

[1] For discussions of this evidence see Child, '20, '21, Chap. II.; '23b; '24, Chap. VII. and literature cited in these publications.

the differential thinning of the blastula wall and the direction of locomotion of the larva all indicate a greater physiological activity at the apical pole, the high end of the gradient, than elsewhere.

The question whether any gradient exists or arises in the entoderm is of interest, but the only method among those used which



FIGS. 12–17.

is available for demonstrating the presence of such a gradient is that indicating different amounts of reduction of permanganate at different levels. After the entodermal cells have formed a definite internal layer in the planula the cleared permanganate preparations usually show a slight entodermal gradient in amount of reduction, as indicated by depth of color. This gradient is similar in direction to the ectodermal gradient but the differences at differ-

ent levels are less than in the latter. The immigration of the entodermal cells singly and their later arrangement in a layer makes it difficult to believe that this entodermal gradient represents differences present in the cells at the time of, or preceding immigration. If it represents real differences in physiological condition, it is probably superimposed on the entoderm by its relation with the ectoderm, the more active regions of the latter determining, perhaps by transmission, greater activity in the adjoining entoderm.

As the planula elongates a second region of high susceptibility, reducing power and rate of staining gradually appears at the basal end (Fig. 15) and from this region a gradient, opposite in direction to the original gradient, develops. The stage at which this second gradient appears varies somewhat in the different species and in different cultures of the same species. Under good conditions it usually appears somewhat before the planula attains its full length. Under slightly depressing conditions it commonly appears somewhat earlier, but under strongly inhibiting conditions its appearance may be completely inhibited. The portion of the planula body over which the second gradient extends during the free swimming stage varies considerably, being usually one fifth to one third the total length. Undoubtedly the degree of development of this second gradient before attachment depends on the stage at which it first appears and on the condition of the larva.

This second gradient is the first indication of the hydranth-stem axis. As the stage of attachment approaches the activity of the original apical region decreases and the original apico-basal gradient becomes less distinct, while the new gradient at the basal end becomes longer and more distinct. During this period the swimming activity decreases and sooner or later attachment by the original apical end occurs (Fig. 16), a perisarc is secreted and elongation of the basal region to form the hydranth and stem begins (Fig. 17).

Thus far no indications of the original apico-basal gradient have been observed at the stage of Fig. 17. Apparently the only gradient present is the secondary gradient which originated at the original basal end. The objection may be raised that the presence of the perisarc and the attachment of the original apical end

protect this region from the action of external agents. This, however, is not the chief factor in the low susceptibility and reducing power of this region, for when these stages are scraped from the substratum the attached surface is often left exposed, but even then the original apico-basal gradient does not appear. As a matter of fact, various experiments on susceptibility and reducing power of naked protoplasmic surfaces and those covered by perisarc have shown that, except when the perisarc is old and very thick, it is rapidly penetrated by most agents. The very thin perisarc of stages such as Figs. 16 and 17 is penetrated rapidly by $KMnO_4$ and the agents used to determine susceptibility

The tip of the growing stem which represents the high end of the secondary gradient gives rise to the first hydranth (see Fig. 8). This hydranth itself shows a marked apico-basal gradient which is a part of the hydranth-stem gradient. Each tentacle, originating as a localized region of growth on the hydranth body, represents a gradient with the high end at the tip. The tentacle gradient apparently originates in the localization of the region of growth giving rise to the tentacle. This region is not sharply defined, but its activity decreases peripherally from a central area and this decrease evidently constitutes the beginning of the tentacle gradient. The gradients of numerous other axiate organs undoubtedly arise in the same way. The localization of a region of growth giving rise to a tentacle must be determined by correlative factors in the developing hydranth. The tentacles arise at a certain level of the polar gradient and at a certain distance from each other. Each growing tentacle region, once localized, apparently dominates a certain area as does a growing plant bud and determines the course of development of this area. From this viewpoint the tentacle is a gradient in a specialized part which originates at a certain body level in reaction to the polar gradient of the body. In the species serving as material for this paper the tentacles of a circle are apparently not simultaneously determined or localized but the factors concerned in their localization are obscure.

The Physiological Significance of the Various Methods.

The first question which arises is whether the axial differences in susceptibility to the various agents used result merely from differences in permeability of the limiting surfaces of the cells. That axial differences in permeability to some, if not to all agents, corresponding more or less closely to the gradients observed, do exist is probable. But that such differences are not the only, nor the fundamental factors in the gradients is indicated by various facts, some of which require brief consideration in connection with the case in hand. First, an observation made repeatedly on oöcytes of both *Phialidium* and *Stomotoca* teased from the gonad is at least suggestive in this connection. As already noted, the oöcytes are attached during the growth period and when they are teased out of the gonad rupture of the cytoplasm occurs at the attached pole and, although the injured region closes within an hour or two, the intact plasma membrane present over other parts of the surface is absent in this region for some time and granules or droplets are often given off into the water. But even while this cytoplasmic region is exposed, it does not become the high end of the susceptibility gradient or the reduction gradient. That is at the opposite pole, in spite of the fact that the agents must penetrate the intact membrane there in order to produce their effects. The only effect of the exposure of the cytoplasm at the attached pole is a little superficial cytolysis or reduction. This absence of relation between the polar gradient of the oöcyte and the injury at the attached pole is striking.

Second, as regards the action of the various agents used to demonstrate the gradients by cytolysis, it is important to note that the cytolytic gradients are the same for agents such as methylene blue and neutral red which penetrate living membranes readily and kill by accumulation within the cells and for those such as $HgCl_2$ which, in the concentrations used, alter and kill the cell surfaces rapidly and so destroy such differences in permeability as may have existed. Again, the cyanides or the CN ion apparently penetrate cells readily and produce their effects largely within the cells, according to recent views by inhibition of the catalyst of oxidation. As a matter of fact, susceptibility to KCN has been shown in various ways to be, within certain

limits a very delicate indicator of physiological condition and particularly of rate of cell respiration. When susceptibility gradients are found to be essentially the same for different agents which act on living protoplasm in ways as different as KCN, $HgCl_2$ and the vital dyes in the concentrations used, it seems impossible that the differences in susceptibility can be due simply to differences in permeability. In this work on the hydroids the use of a large number of agents for lethal susceptibility was regarded as unnecessary for several reasons: first, because differential susceptibility to KCN is believed to be a valuable indication of quantitative differences in physiological condition which in most, if not in all protoplasms, are associated with oxidative processes; second, because the data obtained by different methods, lethal susceptibility, reduction and modification of development, the last to be considered in a later paper, all agree as regards the physiological gradients indicated; and third, because in the light of earlier work on many different forms, with the methods used here and with other methods, the data on hydroid development appear merely as additional evidence in agreement with that already at hand.

And finally, the physiological gradients demonstrated experimentally correspond to differences in rate of the developmental processes. The high ends of the gradients are the most active regions. Differences in permeability are in all probability concerned in the differences in activity, but if this is the case, permeability is merely one factor in the complex of metabolic and other factors which constitute physiological condition.

The basis of the general relation between physiological condition and susceptibility has been discussed elsewhere. At present it need only be pointed out that it does not depend on the particular method of action of a particular agent, but is apparently a relation between a certain degree of disturbance of any kind and the rate of change characteristic of the system disturbed. To a degree of disturbance adequate to bring about disruption or irreversible change of the system in course of time, the more active region is more susceptible because its own activity becomes a factor in producing the total or final effect (Child, '23*b*).

As regards the gradients indicated by reduction of $KMnO_4$, it has already been noted that they appear both as gradients in rate

of reduction indicated by rate of appearance of the brown color and as gradients in amount of reduction indicated by depth of color when the reaction proceeds to completion in excess of the agent and the preparations are dehydrated and cleared. The possibility that a differential permeability along the axis, either of the plasma membrane of naked stages, or of the perisarc in stages possessing it, is a factor in the gradient in rate of reduction need not be denied. It is highly improbable, however, that such differences in permeability are fundamental factors in the gradients of naked stages for $KMnO_4$ in the concentrations used penetrates rapidly and even alters the visible aggregation of the protoplasm before the reduction gradient becomes distinct. Any differences in permeability which may have been present must be destroyed early in its action. Moreover, the possibility that differential permeability determines the gradient in amount of reduction as indicated by depth of color due to oxides precipitated throughout the cell or cells, seems to be eliminated. And finally, the fact that the gradients in rate and in amount of reduction are the same for a particular stage and are also identical with the gradients in susceptibility indicates clearly enough that the different methods of demonstrating the gradients are merely different ways of making evident one aspect or another of the general quantitative gradation in condition of the living protoplasmic system.

That the gradients indicated by reduction of $KMnO_4$ are closely associated with the processes of living in the protoplasm is further shown by the fact that individuals of various stages killed in various ways and then placed in permanganate reduce much less of the agent than living individuals and show, either no traces of axial gradients, or in some cases slight traces for a short time after the action of the killing agent. For example, when developmental stages are placed in alcohol, $HgCl_2$ or various other agents used for histological purposes, then washed and brought into $KMnO_4$ after various periods in the killing agent, it is found that a few seconds in most agents is sufficient to obliterate in large measure the differences on which the reduction gradient depends, though slight traces of a gradient may persist in some cases, even for several hours. In material carried up to 80 per cent. alcohol,

returned to water after a day or two and then brought into permanganate no traces of the reduction gradient have thus far been found. The persistence of traces of the gradients for a short time after action of a fixing agent can scarcely be due to the persistence of a permeability gradient, even if such were present in the living animal. Another interpretation appears more probable, viz., that complete inactivation of the oxidizing enzymes does not always occur at once in the fixing agents, consequently traces of the original differential may persist for a short time. Individuals killed before exposure to permanganate never become opaque black but remain yellow or brownish indefinitely in excess of the agent. Evidently killing before exposure decreases greatly the reducing power of the protoplasm.

The gradients of differential staining with neutral red and the higher concentrations of methylene blue are visible only during the early stages of staining in living material. As the staining of living material progresses the regional differences in depth of color gradually disappear and, so far as the eye can distinguish, the staining becomes uniform throughout long before the depth of color is sufficient to obscure differences. But if staining is continued until cytolysis occurs the cytolytic gradients are the same for any particular developmental stages as those observed with other agents.

The differential staining gradients may represent primarily the differences in permeability of different regions to the dyes, but if appearances can be trusted these differences in permeability disappear as the toxic action of the dyes progresses and in spite of the fact that all regions seem to be stained uniformly, the regions which are more susceptible to other agents and which show greater reducing power are more susceptible to these dyes. Here as in other forms, such differential permeability as may exist appears to be merely one aspect of the differences in physiological condition which constitute the gradients.

In the case of methylene blue the ability of the living protoplasm to reduce and decolorize the dye rather rapidly introduces another factor and makes possible demonstration of the gradients as gradients in rate of reduction and decoloration, particularly in the blastula, planula and later stages, in which the rate of metab-

olism is higher than in early stages. The reduction gradients with methylene blue appear only in relatively low concentrations or after removal to water before staining has progressed too far, but the concentrations and periods of staining suitable for this purpose differ with the different species. For example, the planulæ of *Phialidium* show differential staining with the high regions of the gradient or gradients most deeply stained in the early stages of staining with methylene blue 1/1000, but in 1/5000 scarcely a trace of differential staining occurs, the whole larva staining more or less uniformly. In 1/20,000 there is no appreciable staining during the first two hours of exposure, but after six hours many planulæ show a reversed staining gradient. In the shorter planulæ the depth of staining is least at the apical end of the larva and increases basipetally; in the longer planulæ, in which the second gradient is already present, both ends are slightly or not at all stained and the depth of color increases toward the middle region. Similar reversal of the staining gradient has been observed with low concentrations of methylene blue in various multicellular forms and in ciliate infusoria, including *Paramecium*. These reversed staining gradients in low concentrations result from reduction of the dye to the leuco-compound in the protoplasm. Below a certain concentration, which must be determined experimentally for each species, the higher levels of a gradient reduce the dye as rapidly, or almost as rapidly as it enters and more rapidly than lower levels. Consequently the depth of staining is least at the high end and increases toward the low end of a gradient. Even with concentrations high enough to stain the high regions of the gradients more deeply the reduction gradients often appear when the animals are returned to water before the staining has progressed far. Apparently the reduced dye is toxic, for death and cytolysis finally occur in concentrations so low that the high regions of the gradients which disintegrate first are only slightly or not at all stained. It is improbable that the toxicity of such concentrations, *e.g.*, 1/20,000, can be due to impurities.

Conclusion.

The evidence from the study of the gradients during development agrees with the data of observation in showing, first that

the polarity of the oöcyte becomes the polarity of the early planula, and second that a complete reversal of polarity occurs in the course of transformation of planula into hydroid. A simple interpretation of this reversal is possible in terms of physiological gradients. The hydranth-stem axis apparently arises as a bud from the original basal end of the planula. The appearance of the second gradient at the basal end as the planula elongates suggests that the new axis arises through physiological isolation of this basal region from the dominant apical region in consequence of increase in length. Decrease in activity of the apical region, which is indicated by the lower susceptibility of these, as compared with earlier stages of the planula, may also decrease the range of dominance and so play a part in the physiological isolation of the basal region. In these larval stages the range of dominance is undoubtedly very short because the mechanisms of transmission are rudimentary (Child, '15, pp. 149–151) and physiological isolation may occur at a very short distance from the dominant region. In consequence of the isolation the cells at the basal end become more active and lose whatever differentiation they have attained, while the activity of other parts continues to decrease with the progress of differentiation, until the region originally basal and least active becomes the most active region of the body. With further development and with the differentiation of a rudimentary nervous system the range of dominance increases, as in the development of other forms, and the hydranth which arises at the high end of the secondary gradient comes to dominate the whole body, until further increase in length leads sooner or later to physiological isolation again and budding begins. The reversal in direction of polarity in the hydranth-stem axis apparently results from the fact that in the course of elongation of the planula the extreme basal region is the first part to be isolated and to become more active. This precedence makes it the high end of the new gradient. It is evident from the work of many investigators that polarity in the hydroids is extremely labile and readily altered by external conditions. Every hydroid bud represents a new gradient and, so far as can be determined, this bud which forms the first hydranth differs from those which later give rise to branches only in its position at the

opposite end of the polar axis from the original dominant region. In that respect it is similar to the "axial heteromorphoses" which are characteristic features of the normal budding and of the reconstitution of stem pieces in *Tubularia* and many other hydroids (Child, '07*a*, *b* and literature there cited; '15, pp. 91, 92, 133–37). The term "heteromorphosis" is merely descriptive and leaves us without any interpretation of the phenomena concerned. So far as analyzed physiologically, all cases of so-called axial heteromorphosis are essentially cases of budding which involve the origin of a new polar gradient. Actually every hydroid bud and every bud in a multiaxial plant is as truly a heteromorphosis as are these cases of buds arising at the basal end of an axis. All such buds represent the determination of new physiological axes and the conditions, external and internal, determine in each case where the new axes shall appear.

In some of the tubularian hydroids, *e.g.*, *Corymorpha* (Torrey '07, and my own observations), the hydranth-stem axis arises from the original apical end of the planula and although the secondary gradient appears sooner or later at the basal end, physiological isolation is not complete and the secondary gradient gives rise, at least in its earlier stages only to stolon axes, which are inhibited axes (Child, '23*a*). In these forms, then, the primary gradient persists as the gradient of the hydranth-stem axis.

Summary.

1. The axial gradient and its changes during development were demonstrated in *Phialidium* from oöcyte to hydroid stage by differential susceptibility to various agents, differential reduction of $KMnO_4$ and methylene blue and differential vital staining. Developmental stages of *Gonothyræa* and *Stomotoca* served as comparative material. The results of the different methods agree among themselves and with the observed facts of development.

2. In *Phialidium* and *Stomotoca* the polarity of the oöcyte is indicated by a gradient with high end at the free pole, low end at the attached pole. This gradient is directly related as regards direction to the differential exposure of the oöcyte in the gonad and it is suggested that the polarity of the oöcyte is determined by this differential exposure.

3. The high end of the oöcyte gradient becomes the apical pole of the egg at maturation and in early cleavage stages. During later cleavage a gradient is continuously present, but absence of landmarks makes complete demonstration of its identity with the gradient of earlier stages impossible, though there is no reason to doubt that it is the same. From the beginning of locomotion on the identity of the gradient is certain, the high end being the apical, the low end, the basal pole of the larva.

4. As the planula elongates a second gradient opposite in direction to the primary gradient appears at the basal end of the larva and gradually extends toward the apical end. Soon after this gradient appears the planula of *Phialidium* and of *Gonothyræa* attaches itself by the original apical end and the second gradient becomes the hydranth-stem axis. Attachment of the planula of *Stomotoca* was not observed.

5. The origin of this second polarity, opposite in direction to the first, is interpreted as a result of physiological isolation of the basal end of the planula in consequence of increase in length. From this viewpoint the hydranth-stem axis represents a process of budding not essentially different physiologically from other processes of budding in hydroids and various other forms.

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STUDIES OF COAT-COLOR AND FOOT PIGMENTATION IN SUBSPECIFIC HYBRIDS OF *PEROMYSCUS EREMICUS*

F. B. SUMNER AND R. R. HUESTIS.

I. INTRODUCTION.

In several previous papers [1] the senior author has reported upon inheritance, variation, correlation and allied phenomena in crosses between several subspecies of *Peromyscus maniculatus*. A considerable number of measurable characters were dealt with in the course of these studies, some of these relating to the absolute or proportional size of various bodily parts, others to the pigmentation of the hair and skin.

It was early evident not only that the characters distinguishing two subspecies, taken collectively, did not depend upon single mendelian allelomorphs, but that the same was true of each of the various subspecific differences, taken separately. So far as studied, all of these characters followed the "blending" type of inheritance. If they were dependent upon mendelian factors at all, several or many of these were concerned in the production of each character difference. Moreover, studies of correlation showed that in many cases these separate characters were inherited independently of one another.

About three years ago, the junior author began the study of another species of *Peromyscus, P. eremicus,* a form differing sufficiently from *P. maniculatus* to be assigned to another subgenus.[2] Two subspecies were dealt with, *P. eremicus eremicus* and *P. e. fraterculus*. The parent stock of the former race was trapped in Death Valley, California, in 1920, the latter animals were taken in the neighborhood of La Jolla. In appearance, these two geographic races differ in the same manner as do desert and coast-dwelling representatives of various other species of rodents. The

[1] *Journal of Experimental Zoölogy,* Oct., 1923, and papers therein cited.

[2] For a brief summary of the results of these studies see Huestis, *Proc. Nat. Acad. of Sci.,* 1923. A fuller account is now in press.

difference is about as great, for example, as that between *P. maniculatus sonoriensis* and *P. maniculatus gambeli.*

The material comprised in these studies included 208 specimens of the parent races, *eremicus* and *fraterculus,* 199 F_1 and 147 F_2 hybrids. No bodily measurements were made of these mice, nor were skeletons saved. Skins were, however, prepared according to the method long practiced by the senior author.[1] Later, the feet were removed from the dried skins for a study of the pigmentation of the soles. After removal, they were first soaked for a day in distilled water, next transferred to 70 per cent. alcohol, then to equal parts of alcohol and glycerine, and finally to pure glycerine. After this treatment, the feet were graded according to a scale previously adopted by the senior author.

The series of skins upon which these studies were made were superior in two respects to those earlier employed by us in the study of *Peromyscus.* In the first place, the numbers were larger, both for the pure races and the hybrids. Secondly, the animals were skinned at a somewhat greater age, eight months being adopted as a minimum instead of 6½ months, as had been the previous practice.[2] Thus the hair of these mice seldom showed any traces of the second molt, having passed into the third (first "adult") pelage.[3]

From these prepared pelts small samples of skin, with the accompanying hair, were removed with a sharpened cannula, having a diameter of about 1.5 millimeters. The hairs were subjected by the junior author to quantitative determinations of two sorts. (1) They were grouped, on the basis of their size and pigmentation, into four somewhat arbitrarily distinguished classes, and the number of each class comprised in the sample was counted. (2) The length of the subterminal ("agouti") band and of the terminal region, distal to this, were measured for ten of the "class A" hairs in each sample. Likewise, the total length of the long-

[1] Sumner, *Journ. Exp. Zoöl.,* Aug., 1909 (p. 101); described more fully by Collins, *Journ. Exper. Zoöl.,* Aug., 1923 (p. 48).

[2] A few which died at an age less than 8 months were included, provided that the hair of these showed no trace of molting. On the other hand, some others were several months older than this minimum age, owing to their having been kept for breeding purposes.

[3] At the age adopted for the earlier series (6½ to 7 months) a certain proportion of animals had not fully completed the second molt.

est hairs of the sample, *i.e.*, the maximum hair length of this region, was determined in each case.

It was already known that the general coat color of the individual depended upon the proportional number of different types of hairs in the pelage, as well as upon the relative length of the subterminal yellow band in the "agouti" hairs and some other characters. If, therefore, the color differences between these various geographic races of mice were due to differing combinations of mendelian unit-factors, such factors would be more likely to reveal themselves in the hereditary behavior of these more elementary characters than in that of the mass effects, as shown by photometer readings of considerable areas of the pelage. To what degree, if at all, this theoretical expectation has been realized, will be discussed later.

In the following pages, we shall deal with these mass effects first, before proceeding to a consideration of the various microscopic hair characters which form their basis. This seems the more logical order to follow, despite the fact that in the case of this particular series of skins the microscopic studies preceded the photometric ones.

II. General Coat Color as Determined by the Tint Photometer.

The Hess-Ives Tint Photometer and its use in the study of mammalian pelages have already been discussed briefly by the senior author in several previous papers.[1] Pending the preparation of a more adequate account of this procedure, a very brief restatement of the technique employed must suffice.

A rectangular area of the pelage, together with an equal area of a "standard" white, are viewed in juxtaposition through three successive color screens (red, green and blue-violet). In each case, the light from the standard white is cut down by a shutter until the luminosity (and necessarily the color as well) is equal to that of the object under examination. The readings of the lever controlling this shutter, which are expressed in hundredths, furnish the data from which the color and luminosity values here employed are computed.

[1] For most complete statements see Sumner and Collins, *Journ. Exp. Zoöl.*, Oct., 1922, pp. 289, 294.

The values employed in the studies thus far made are the percentages of "black," "white" and "color" in the sample. For reasons which we shall not justify here, the difference between the highest reading (always red, in our material) and 100 is regarded as the percentage of "black"; the lowest reading (blue-violet, in our material) is regarded as being likewise the percentage of "white"; while the balance (difference between the sum of these two and 100) is regarded as the percentage of "color." By "color" is here meant the free color, *i.e.*, that which remains of the two higher readings after deducting the amount regarded as combining to form white light. The spectral position of this "color" is not, of course, given in any direct way by these readings. It reaches its maximum, in all cases, somewhere between red and yellow, its nature being indicated crudely by the ratio between "free" red and green (R:G ratio). This ratio is high in reddish skins, low in yellowish ones.[1]

In the present studies, two dorsally situated and symmetrical areas of the pelage were selected, one being in the shoulder region, the other a little anterior to the base of the tail. The means of the values for the two areas were employed in the computations, and each value was likewise based upon two readings, made upon different days.

In Table I. are given the means and standard deviations for each of these "characters," the two parent races and the two hybrid generations being considered separately. The number of individuals here given is in each case smaller here than that employed by Huestis for the study of microscopic hair characters. This is because certain pelts which could be used for the latter purpose were not suitable for the former, owing to injury or to molting within the areas under examination.

The salient facts shown in the foregoing table are as follows:

1. As was evident from a casual inspection of skins, *P. e. fraterculus* is a considerably darker race than *P. e. eremicus*. This is indicated by a higher percentage of "black" in the former and by the lower percentages of "white" and "color."

[1] To illustrate: suppose that the readings for red, green and blue-violet are 25, 15 and 11, respectively We have in this case, 75, 11 and 14 respectively, as the percentages of black, white and color. The R:G ratio is 3.5.

TABLE I.

	Number.	Black.		White.		Color.		R : G Ratio.	
		Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.
eremicus	92	77.56 ± .14	1.97 ± .10	13.41 ± .07	1.04 ± .05	9.03 ± .09	1.29 ± .06	2.49 ± .013	.18 ± .009
fraterculus	93	84.02 ± .09	1.36 ± .07	9.67 ± .06	.80 ± .04	6.31 ± .07	.99 ± .05	2.59 ± .014	.21 ± .010
F_1 hybrids	177	79.56 ± .08	1.50 ± .05	12.32 ± .04	.88 ± .03	8.12 ± .05	1.04 ± .04	2.56 ± .009	.18 ± .006
F_2 hybrids	132	80.44 ± .12	2.01 ± .08	12.22 ± .06	1.07 ± .04	7.34 ± .07	1.27 ± .05	2.44 ± .010	.17 ± .007

2. The R:G ratio is slightly, though perhaps significantly, higher in *fraterculus* than in *eremicus*. In other words, the darker race seems to be relatively redder, the paler race relatively yellower. It was earlier observed that a dark race (*rubidus*) and a pale race (*sonoriensis*) of another species (*maniculatus*) differed in this same direction, though to a greater extent.

3. The mean values for the F_1 generation of hybrids lie, in each case, between the values for the two parent races, though they are not in any case midway in position.

4. The mean values for the F_2 generation differ somewhat from the F_1 values, but they are none the less intermediate between those of the parent races with the exception of the figure for the R:G ratio.

5. The standard deviations for the F_2 generation exceed those for the F_1 in the case of black, white and color, the differences being 5.4, 3.8 and 3.6 times their probable errors, respectively. There is no such increase of variability, however, in the case of the R:G ratio, the standard deviations for which are approximately equal in the two generations. It will be noted that the three former "characters" differ rather widely in the two parent races, whereas the difference in the latter case is slight.

The statistical certainty of the color differences between the subspecies which were crossed, and the fact that these differences persist, apparently undiminished, after several generations in captivity, show that they are largely genetic in their nature. The same is not so evident with respect to the individual differences within a single race or within a group of hybrids of similar origin. Observation shows that part of these differences are due to age or to condition of molt; while season is perhaps likewise responsible to a certain extent. Previous studies [1] have shown, however, that a certain fraction of this individual variability has a genetic basis. For the present material, coefficients have been computed, indicating the degree of parent-offspring correlation with respect to the four values employed in our computations (black, white, color and the R:G ratio). The resulting figures are given in the following table.

[1] Sumner, *Journ. Exp. Zoöl.*, Oct., 1923.

TABLE II.

Parents and Offspring Correlated.	Number of Parents.	Number of Offspring.	Black.	White.	Color.	Mean of Preceding Three.	R : G Ratio.
eremicus—F_1 .	33	153	+.146±.068	+.197±.067	+.283±.064	+.209	−.047
fraterculus—F_1	18	176	+.321±.062	+.220±.065	+.331±.061	+.291	+.114
F_1♂—F_2	30	132	+.274±.069	+.272±.069	+.216±.071	+.254	−.002
F_1♀—F_2	40	126	+.188±.071	+.210±.071	+.199±.071	+.199	+.094

These figures show that part of the individual variability in coat color is genetic. The correlation is, however, rather low, the mean of the 12 figures for "black," "white" and "color" being only 0.28. This indicates that a large proportion of the variability is due to non-genetic causes.

Furthermore, these and previous data make it doubtful whether individual differences in the R:G ratio are inherited at all, despite the fact that two races may differ rather widely from one another in respect to this character.

III. MICROSCOPIC HAIR CHARACTERS.

The junior author has already published a brief account of his findings with respect to certain hair characters which he has been able to determine quantitatively with the microscope.[1]

Table III. gives the mean values as well as the variability of seven "characters" in the two parent races and in the F_1 and F_2 generations of hybrids. It is evident that the four mean values comprised in the first half of this table are not independently variable, since their sum is in every case 100. The classification of hair types here included is somewhat arbitrary, as the various classes intergrade completely with one another. The hairs of these animals may be distinguished as banded (classes *A*, *B*, *C*) or as non-banded (*D*). In the latter very dark ("sepia") pigment is present throughout the entire length of the hair. In the former, this dark pigment is interrupted, toward the distal end, by a yellow cross band of varying extent, giving rise to the condition known to geneticists as "agouti."

[1] *Proc. Nat. Acad. Sci.*, Oct., 1923.

TABLE III.

	Number.	Percentage *A* Hairs.		Percentage *B* Hairs.		Percentage *C* Hairs.		Percentage *D* Hairs.	
		Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.
eremicus	108	50.39 ± .34	5.23 ± .24	29.43 ± .31	4.72 ± .22	8.39 ± .22	3.37 ± .15	11.52 ± .20	3.01 ± .14
fraterculus	100	59.27 ± .30	4.44 ± .21	18.32 ± .22	3.23 ± .15	9.82 ± .16	2.41 ± .11	12.27 ± .21	3.13 ± .15
F_1 hybrids	199	56.42 ± .23	4.73 ± .16	23.37 ± .18	3.71 ± .12	9.15 ± .13	2.74 ± .09	10.71 ± .16	3.43 ± .12
F_2 hybrids	147	56.25 ± .31	5.48 ± .22	22.95 ± .21	3.76 ± .15	9.04 ± .17	3.07 ± .12	11.52 ± .21	3.80 ± .15

TABLE III—*Continued.*

	Total Length of Hair in Mm.		Length of Agouti Band in Tenths of a Mm.		Length of Distal Tip in Tenths of a Mm.	
	Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.
eremicus	12.40 ± .06	.87 ± .04	14.21 ± .09	1.41 ± .06	3.35 ± .02	.39 ± .02
fraterculus	11.08 ± .04	.61 ± .03	8.38 ± .06	.93 ± .04	4.36 ± .03	.38 ± .02
F_1 hybrids	11.23 ± .03	.67 ± .02	12.06 ± .06	1.29 ± .04	3.78 ± .02	.38 ± .01
F_2 hybrids	11.48 ± .04	.79 ± .03	11.32 ± .10	1.87 ± .07	3.90 ± .03	.50 ± .02

Of the banded hairs, class *A* includes those which are slender throughout (they are also relatively short), while class *B* includes those which broaden out through the region of the subterminal band. Class *C* comprises hairs which are intermediate between *B* and *D*, and for which the creation of a separate class has seemed justifiable.

In the lower section of Table III. will be found the "total length," together with the lengths of the two distal subdivisions of the hair, all of these values being based upon micrometer measurements. The first of these figures represents the length of the longest hairs in each sample, the two last are means derived from the measurement of ten "*A*" hairs in each sample.

It will be noted that the proportion of *D* (all-black) hairs is slightly greater in the darker race (*fraterculus*) than in the paler race (*eremicus*), but that the chief difference between the two lies in the greater proportion of *B* (broad "agouti") hairs in *eremicus*. This is doubtless partly responsible for the paler and more highly colored pelage of the latter subspecies. As regards the slender, banded hairs of the *A* class, the relations are reversed, the darker race (*fraterculus*) having proportionately more than the paler race. Owing to the relative shortness and slight width of these hairs, they contribute, however, much less to general color effect of the pelage.

Eremicus appears to have slightly longer hairs than *fraterculus*, but the fact of chief interest revealed by the linear measurements is the considerably greater mean length of the "agouti" band in this race. The figures here given are based upon hairs of the "*A*" class, but similar relations would have held in the case of the "*B*" hairs,[1] and this difference in the length of the yellow subterminal band is without doubt another of the important factors which are responsible for the color differences between these two races.

Further scrutiny of the table reveals the fact that in the case of six out of seven of these "characters," the mean values of both hybrid generations are intermediate between the values for the parent races. The single exception relates to the percentage of *D*

[1] It was less practicable to employ these in the present measurements.

hairs, a character in respect to which the parent races differ but slightly.

From the standpoint of current genetic theories, the figures of chief interest in this table are those relating to the relative variability of the F_1 and F_2 generations of hybrids. It will be noted that in all of the seven cases under comparison the standard deviation is larger for the second generation than for the first, although in some cases the difference is not statistically significant. The probability quotient (difference ÷ probable error) ranges from 0.3 to 7.2, the mean of the seven figures being 3.3. It is to be remarked that the increase of variability is most certain ($D/E = 7.2$) in respect to one of the characters (agouti band) which differ most widely in the two parent races. But it must likewise be noted that the increase is quite non-significant ($D/E = 0.3$) in the case of the other most distinctive racial difference (percentage of *B* hairs.)

It is of interest to compare the evidence for segregation (so far as this may be inferred from the F_2 variability), as shown by this series of determinations and by those made with the tint photometer. Omitting the figure for total length of hair, which has little to do with pelage color, we have the following $\frac{D}{E}$ quotients for the "characters" thus far considered:

B hairs	0.3	White	3.8
D "	1.9	Black	5.4
C "	2.2	Tip	5.5
A "	2.8	Agouti	7.2
Color	3.6		

Here it will be seen that the mean of the quotients for the four classes of hairs is 1.8, that for black, white and color being 4.3, and that for the two measured segments of the individual hairs being 6.3. So far as may be judged from the foregoing figures, it can hardly be said that these microscopic hair characters show, on the whole, any greater evidence of segregation than do the color values for the aggregate pelage. The figure for black is exceeded (significantly) by only one other figure, that for the agouti band, while the figures based upon the hair counts are the

lowest of the series. If we accept the degree of segregation[1] as a safe index of the number of genetic factors underlying a given character, we must conclude that only one of the foregoing characters (agouti band) is more elementary, genetically speaking, than the crude value for "black" in the pelage. While the data at hand do not warrant any such simple inference, we may safely affirm that microscopic analysis has thus far helped us little in the search for unit factors underlying the coat color of *Peromyscus*.[2]

That we are here dealing in part with genetic differences is plain from a consideration of parent-offspring and fraternal correlations based upon these characters. The following figures are the means for six of the characters, the coefficients for "tip" not having been computed:

Parent-offspring

Eremicus — F_1	+ .302
Fraterculus — F_1	+ .267
F_1 ♂ — F_2	+ .254
F_1 ♀ — F_2	+ .205

The mean of these figures is 0.257, being thus somewhat greater than the mean of the figures (0.238) for black, white and color. Correlation is not equally strong for all of these characters. The mean of the figures for the *A*, *B*, *C*, and *D*, hairs is 0.227, that for total length being 0.314, and that for the agouti band 0.319. These figures suggest that the proportion of non-genetic variability is considerably greater in the case of the hair counts than in the case of the linear measurements, a fact of possible significance in connection with the inferior evidence for segregation shown by the former characters.

It is to be noted that the mean correlation between the pure-race parents and their F_1 offspring (0.284) is somewhat greater than

[1] Strictly speaking, these quotients indicate the probability of the existence of significant differences, rather than the amount of such differences. With equal numbers and equal variability, however, they are proportional to the magnitude of the differences.

[2] A circumstance tending to diminish the validity of these comparisons is the varying degree of accuracy of the technique upon which the measurement of the various characters rests. The greater the observational error, the greater, of course, will be the proportion of the total variability which is due to non-genetic causes. Concerning the relative magnitude of this factor in the different cases, we have little basis for opinion.

that between the F_1 parents and their F_2 offspring (0.229).[1] This condition is paralleled by that shown in Table II. for black, white and color. It might be explained as due to the segregation of factors in the F_2 generation, a process which would obviously weaken the correlation between members of this generation and their parents. In this connection it is also worth noting that the coefficient of fraternal correlation is higher for the F_1 generation (.356) than for the F_2 generation (.328), a fact which is in accord with the same interpretation.[2]

IV. Foot Pigmentation.

The feet, prepared as above indicated, were graded according to the depth of pigmentation in the skin of the soles. In the present series, both feet of each specimen were independently graded, the mean of the two recorded values (in the exceptional cases where they differed) being employed. The following figures give the means and standard deviations for the parent races and the two generations of hybrids, the numbers being approximately the same as in the case of the skins:

	Means.	St. Dev.
Eremicus	$0.45 \pm .03$	$0.48 \pm .022$
Fraterculus	$2.02 \pm .05$	$0.79 \pm .038$
F_1 hybrids	$1.65 \pm .03$	$0.60 \pm .020$
F_2 hybrids	$1.72 \pm .04$	$0.71 \pm .028$

The three salient facts shown by the foregoing figures are (1) the much heavier pigmentation of the feet of *P. e. fraterculus*, as compared with *eremicus*, the mean difference being somewhat greater than that between *P. maniculatus gambeli* and *P. m. sonoriensis* from the same two localities; (2) the intermediate position of both hybrid genertions, which, however, average darker than the mean of the two parental figures and (3) the greater variability of the F_2 generation as compared with the F_1. We thus have much the same situation here as in the case of the other characters which have been discussed.

[1] It should be added, however, that this relation holds for only 3 of the 6 "characters," taken singly.

[2] The circumstance that the F_2 broods resulted, to a considerable extent from the mating of sibs would tend to increase the second of these figures, and thus to diminish the difference between the two hybrid generations in respect to fraternal correlation.

In connection with certain bone measurements, the authors showed that a higher degree of bilateral asymmetry occurred in the F_2 generation of subspecific hybrids than in the F_1 generation.[1] We have accordingly compared the two hybrid generations of this cross in respect to asymmetry in the pigmentation of the feet. The number of cases in which the right and left feet were assigned to different grades was, however, very small. Four such cases occurred among the 197 F_1 individuals and five among the 147 F_2 individuals. The larger proportion among the latter cannot be regarded as significant.[2]

The following coefficients were computed, expressing the degree of correlation between parents and offspring in respect to foot pigmentation:

Eremicus — F_1	+ .097
Fraterculus — F_1	+ .159
F_1 fathers — F_2	+ .342
F_1 mothers — F_2	+ .273

The low degree of correlation between the pure race parents and their F_1 offspring (mean = .128) is believed to be due to the wide range in the age of the parent stock, in comparison with the hybrid animals, which were almost homogenous in this respect. Independent data have shown us that young animals have darker feet, and that the depth of pigmentation decreases with age, at least up to a certain point. Variability due to this cause would of course weaken correlation.

Fraternal correlations for this character are $.358 \pm .043$ and $.198 \pm .054$, for the F_1 and F_2 generations respectively. The difference here is only 2.3 times its probable error.

V. Intra-racial Correlations.

A considerable number of coefficients of correlation have been computed in order to ascertain to what degree certain characters tend to vary together among the individuals of a single race. Most of these need not concern us in the present paper. Some of them are of little biological interest, and some, indeed, express

[1] *Genetics*, Sept., 1921.

[2] The negative results in the present case may well be due to the relative crudity of the scale employed. The deviations from symmetry recorded for the paired bones were only detected by exact measurements.

quantitative relations which are merely due to mathematical necessity.

The point of chief interest, perhaps, is to learn whether two characters, both of which differ in the two races here dealt with, tend to be correlated within the single race. Thus, among other differences, the subspecies *eremicus* is paler and more highly colored than *fraterculus*, has more hairs belonging to the "*B*" class, and less belonging to the "*D*" class, has somewhat longer pelage, a longer subterminal band in the agouti hairs, and a shorter dark segment distal to this, and considerably less pigment in the soles of the feet. To what extent are these character differences associated together within the single race?

Attention has already been called to the fact that in various subspecies of *Peromyscus maniculatus*, as well as in hybrids between these, there seems to be little or no correlation between the darkness of the pelage and the depth of pigmentation of the feet.[1] This is true despite the existence of a marked tendency for darker *races* to have darker feet.

The present studies bear out this conclusion. Separate coefficients have been computed for the males and females of the *eremicus* and *fraterculus* series, as well as for the F_1 and F_2 hybrids. Of these eight coefficients, five are positive, two negative and one 0. The figures range from $-.067$ to $+.253$, the weighted mean of the series being $+.056$. The only figures in the series which are of possible significance are those for *fraterculus*. In any case, we believe that the slight correlation sometimes shown between these two characters is due to heterogeneity in age. Younger mice (even when the pelage is "adult") tend to have slightly darker pelages and slightly darker feet, a circumstance which would insure a certain amount of correlation in a series of mixed age.

In some other cases, however, characters which are associated together as racial attributes have been found to be correlated within the individuls of a race. Thus, positive correlations were found in each of the four groups[2] between the maximum length

[1] Sumner, *American Naturalist*, May–June, 1923.

[2] *Eremicus*, *fraterculus*, F_1 and F_2 hybrids. Here the sexes have been combined in the case of each group.

of the hair and the percentage of *B* hairs (mean = + 1.68), as well as between the length of the agouti band and the percentage of *B* hairs (mean = + .240), while negative correlations were found between length of hair and percentage of *D* hairs (— .187) and between length of agouti band and percentage of *D* hairs (— .221). Such relations suggest the possibility that certain elements of the subspecific complex of characters depend upon factors which are closely linked, or that they may have, in part, a common factorial basis. That this is not true of all the characters distinctive of a subspecies is shown by the case of "black" and foot pigmentation, above referred to, and by various other facts derived from a study of the present and of previous series of animals.

Where correlation between two characters has appeared, whether positive or negative, there appears to be a tendency for this correlation to be greater in the F_2 series of hybrids than in the F_1. While such a condition has not been invariable, the tendency seems to be a real one and not due to accident.[1] Several of these differences taken singly, are of considerable statistical probablity. This phenomenon is, of course, in harmony with the view that the increased variability of the F_2 generation results, in part at least, from mendelian segregation. Whether the correlation of two characters be due to their having some common factorial basis, or to their being dependent on linked factors, the result of segregation would be to increase the correlation between them.

VI. Conclusions.

In a previous paper[2] the authors have discussed the behavior in heredity of small deviations from bilateral symmetry among paired bones. The two outstanding results of that study were (1) that such departures from bilateral symmetry were not inherited, but that (2) we none the less met with the customary increase in the variability of these characters in the F_2 generation of a sub-

[1] In a previous study of *Peromyscus* hybrids (*Journ. Exp. Zoölogy*, Oct., 1923), little evidence was found for such an increase in correlation in the second hybrid generation. Several other correlations which were computed subsequently have rendered such an increase more probable, even for these earlier series.

[2] Genetics, September, 1921.

specific cross. From these facts, the inference seemed reasonable that the increased variability of the second hybrid generation was not in itself trustworthy evidence of mendelian segregation.

From a study of three series of hybrids, derived from two different subspecific crosses, the senior author was later confirmed in the belief that there was little if any proportionality between the increased "spread" of the F_2 generation and the degree of difference between the parent races in respect to the character in question. Cases were pointed out in which a considerable difference between the parent races was associated with little or no increase in hybrid variability, while in other cases, in which the parents did not appreciably differ, the appearance of "segregation" was very marked.

For the present series of hybrids and the small number of characters here dealt with the picture is decidedly different. There is a considerable degree of proportionality between the amount of difference between the parent races and the increased variability of the F_2 generation. In order to test this point more precisely, a correlation table (which it is not necessary to reproduce here) was drawn up. The "characters" under consideration were the twelve which have been dealt with in the present paper. In one column were placed, not the absolute differences between the means of the parental races, in respect to the various characters, but the differences expressed in terms of the variability of these characters.[1] In the other column were placed the differences between the standard deviations of the F_1 and F_2 generations, taken as percentages of increase.[2] Thus the vast differences in the magnitude of the original values (due to differences in the units employed) were largely eliminated.

A graphic presentation of these data revealed the existence of a considerable degree of proportionality between the two series of figures, the only serious discrepancy relating to the behavior of the "*B*" hairs (see above). The extent of the correlation between the two series is indicated by a coefficient of $+.73$. We readily admit, however, the inconclusiveness of a correlation coefficient

[1] Each difference between the parental means was divided by the mean of the parental standard deviations for the character in question.

[2] *I.e.* each difference was divided by the mean of these two standard deviations

based upon 12 pairs of variables, particularly since the characters in queston are not wholly independent of one another, but are themselves, in some instances, rather strongly correlated. On the other hand, it hardly seems likely that this entire correspondence is accidental.[1]

According to the theory of "multiple factors," we should, in general, expect the increase of hybrid variability to be greater, in proportion as the parental difference was greater. Consideration of the simplest possible case, involving a single pair of factors, shows this to be true. Thus, the positive correlations here reported—granting their significance—are in harmony with this interpretation.

To sum up, then, the results of our present inquiries: (1) We find, for a number of measurable characters, affecting the pigmentation of the hair and skin, a blending type of inheritance, both in the F_1 and F_2 generations, following a subspecific cross.

(2) The variability of these characters is, with a single exception, somewhat greater in the second hybrid generation than in the first.

(3) This increase in variability is greatest for the length of the subterminal yellow band, least of all (slightly negative) for the "red:green ratio." The former character is the one which differs most widely in the two parent races, and is likewise most strongly hereditary, as judged by parent-offspring correlations. The latter differs but slightly in the two parent races, while the individual differences within a race do not appear to be inherited at all. With one conspicuous exception, there is a rough proportionality between the amount of the difference between the parent races, in respect to a given character, and the amount of the increase of variability in the F_2 generation.

(4) In general, the coefficients of correlation between F_1 parents and their F_2 offspring are lower than those between the pure-race parents and their F_1 offspring, despite certain elements in our procedure which would tend to increase correlation in the former

[1] A similar calculation, based upon all of the characters of all of the *Peromyscus* hybrids thus far considered (76 pairs of variables) yielded a coefficient of $+0.204 \pm .074$. One of the series, the "Carlotta-Calistoga" lot, yielded, however, a negative coefficient (-0.17).

case. A conspicuous exception to this general rule is, however, to be noted in the case of foot pigmentation.

(5) In general, coefficients of fraternal correlation seem to be lower for the F_2 generation than for the F_1.

(6) Pairs of characters which are correlated in the parent races and in hybrids between these show, in general, a higher degree of correlation in the F_2 generation than the F_1.

(7) Certain elements which contribute to the general pelage color of these mice (aside from those which are obviously interdependent) are found to be correlated with one another, and thus either to have a common genetic basis or at least to depend upon factors which are more or less closely linked. On the other hand, another pair of characters which differ in the same direction in the two races (foot pigmentation and percentage of "black" in the pelage) are apparently not at all correlated within the single race, although individual differences in these characters are known to be partly genetic. Since these two characters are known to have varied together geographically in more than one species of *Peromyscus*, their coincident modification has probably resulted, in some way, from external agencies and not from correlated variations, due to internal causes.

Items 1 to 6, in the foregoing summary, bear upon the theory of "multiple factors," *i.e.*, the view that each of the subspecific differences here discussed is due to the cumulative effect of a number of independent genetic factors, whose effects, taken singly, are similar in their nature. Without entering into an extended discussion, it may be said that the facts here presented conform, on the whole, to the requirements of that theory, and that no facts narrated in the present paper constitute serious objections to it. Items 3, 4 and 6 represent findings which are somewhat at variance with those presented in certain recent papers by the senior author, based upon other hybrid series, and in large degree upon other characters than those here considered.

After careful consideration of the present series, and a further statistical examination of previous material, the senior author is prepared to admit that the case for a "multiple factor" explanation, while far from conclusive, is considerably stronger than he was

earlier disposed to concede. It is important, however, that he should make clear just how much is involved in this admission.

The evidence renders probable the segregation, in the second hybrid generation, of unlike hereditary materials derived from the two parent races. It likewise renders probable the independent assortment of materials affecting the development of various single character differences. Furthermore, it seems to show that each one of these character differences, so far as it is hereditary at all, is influenced by a considerable number of units which segregate independently of one another.

On the other hand, there is nothing in the present evidence to support the view that the entire heritage of an organism is constituted by these segregating "factors." Nor would even a decisive demonstration of the segregation of hereditary units constitute a proof that the segregation was complete or that the units were unchangeable or incapable of influencing one another. One's beliefs in regard to these matters must depend upon the totality of evidence from all sources, and few would claim that at present this evidence is decisive in favor of any particular hypothesis.

In the present state of our knowledge, it is still permissible to suspect that the mendelian "genes" are not the only substances responsible for the hereditary differences between one organism and another. It has frequently been suggested that the genes play the rôle of enzymes in the process of development. But an enzyme must have a "substrate" to act upon, and the end result depends upon the properties of both. Such viewpoint is in harmony with the contention of Loeb "that the Mendelian factors of heredity must have the embryo to work on and that the organism is not to be considered a mere mosaic of Mendelian factors" ("The Organism as a Whole," p. 247).

The results of the present studies are not offered as evidence for such a modified or restricted mendelian viewpoint, though they are in no way inconsistent with it.

ADAPTIVE CHANGES IN SHADES AND COLOR OF *FUNDULUS*.

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I. INTRODUCTION.

Considerable attention has been paid to the changes in shade and color which fishes frequently undergo, and the significance of the phenomenon. The subject is of some theoretical interest and has given rise to a rather extensive literature, though the conclusions arrived at by the various authors are far from unanimous.

There are two important problems involved in these investigations, namely (1) whether fishes simulate the color of the environment and (2) whether they are able to discriminate light of different wave-lengths. In other words, is the spectrum objectively speaking the same for them as for a normal human being or do they see the spectrum as a color blind person does—a gray band of varied intensity. If fishes simulate the background, this indicates discrimination of wave-lengths or color vision, for it has been established that the eye and not the skin as a whole acts as the receptor of the light stimulus. It is, of course, here not im-

plied that they have the same color sensations as a human being has.

Several investigators have attacked the problem of color vision more directly by the method of forming food-associations with definite colors and testing fishes for their ability to distinguish these colors from others of equal brightness. Van Rynberk (1906) gives a critical survey of the earlier work on color changes in fishes and in animals generally, in which the method of food association, as well as that of instinctive response and simulation of background, have been used in testing color vision. The following brief account of the more important recent literature indicates the results obtained by investigators on color changes in various species.

Zolotnitzki (1901) using *Cheironomus* larvæ as food until fishes were accustomed to it, next presented bits of wool of the size and shape of the larvæ but of different colors. The responses to the red were more frequent than to the other colors. He concluded that they discriminate colors, but he disregarded the brightness factor.

Schöndorff (1903) maintained that the earlier workers were in error in asserting that fishes assume the color of the background, but he did so on scanty evidence.

Van Rynberk (1906) found that the flatfish, *Rhomboidichthys* simulates the environment in a striking manner.

Washburn and Bentley (1906) noted that the horned-dace, *Semotilus atromaculatus* discriminates red, green and blue.

Reighard (1908) concluded from his observations on the responses of the gray snapper, *Lutianus griseus,* that it is able to discriminate colors.

Objections have been raised to the conclusion of investigators up to this time, that there was no certainty as to brightness of light for the eyes of the fishes.

Bauer (1910 *a*) experimenting with *Charax puntazzo, Atherina hepsetus L., Box salpa* and *Mugil* sp. concludes that "Wie bei normalen Menschen tritt bei Helladaption, zur Unterscheidung der Helligkeit, die Unterscheidung der Farbenwerte." He observed aversion to red or "Rotscheu" in *Charax* and *Atherina,* and Purkinje's phenomenon in *Mogil* and *Atherina.*

Hess (1910), a leading investigator in the field of color vision in animals, maintains, however, that fishes see the various parts of the spectrum exactly as a color-blind person does at any intensity of light, or as a normal person does at lowered intensity. Bauer (1910 *b*) says this is true only of dark-adapted fishes.

Von Frisch (1911, 1912, 1913) carried out many exact experiments with *Phoxinus* and other species and has come to the conclusion that fishes discriminate colors as well as simulate the background. Hess (1913) maintains, however, that the color of the background has no influence on the color of *Phoxinus*.

Freytag (1914) also found no evidence of color simulation in *Phoxinus*, but subjected the fishes to the stimulus for only twenty-four hours.

Summer (1911) found that certain flatfishes simulate the background not only in shade and color but also in pattern and his photographs given ample evidence that at least these species do so.

Mast (1916) in a very detailed study of the flounders *Paralichthys* and *Ancylopsetta*, proves that these species simulate the background in pattern as well as in shade and color and gives excellent autochromes illustrating the colors assumed.

Reeves (1919) using the method of food association and unlearned responses, and equating the light intensities, concludes that the sunfish (*Eupomotis gibbosus* L.) and horned-dace (*Semotilus atromaculatus*) discriminate light of longer wave-lengths from light of shorter wave-length and from white light, while Ohashi (1921) finds in support of Hess that goldfish and carp are unable so to discriminate, their responses being caused by different light intensities.

During the winter of 1922–23 experiments on *Fundulus heteroclitus* were carried out at the Zoölogical Laboratory, Harvard University, giving positive results which we shall now describe.

II. Adaptive Changes in Shade.

Parker and Lanchner (1922) have recently made tests to ascertain the effect of illumination on the shade of *Fundulus*. They found that the fishes when placed in a white environment represented by a box lined with white paper and illuminated by an incandescent lamp, were of a light shade; those placed in a

black environment represented by a box lined with black paper, were of a dark shade; while those placed in absolute darkness were, contrary to what one would expect, of a light shade. It was also observed that when temporarily blinded they did not exhibit any change in the various environments, showing that the action of light was not a direct one, but indirect, the eye being the receptor.

As a starting point these experiments were repeated and uniform results agreeing with these were obtained.

A test was now made to determine the effect of different colored backgrounds on the shade of *Fundulus*. Four square boxes were lined each with a differently colored paper, one side being left open for the entrance of light. The colored papers matched those named in brackets as given in Ridgway's "Color Standards and Color Nomenclature." The first was lined with light yellow paper (Lemon yellow, Pl. IV., No. 23); the second with red (Nopal red, Pl. I., No. 3, i); the third with green (Scheele's green, Pl. 6., No. 33, i); and the fourth with blue (Bradley's blue, Pl. 4, No. 51).

Fishes were first made to assume the light shade by being left for several days in a white glazed earthenware vessel in the laboratory. From this stock they were selected for experiment. Four specimens were placed in each of four battery jars filled with water to a depth of 10 cm. and the jars placed in the colored boxes. The boxes were illuminated by a white Mazda lamp (100 watts) placed at a distance of 60 cm. from the jars. For purposes of direct comparison all combinations of colored boxes taken two at a time were made, the two boxes and incandescent lamps being screened off from each other. The experiments were all carried out in a large dark room, so that the fishes in each box were under the same environmental conditions, except difference in background. The tests were each of 24-hours duration.

It was found after repeated experiments that the specimens from each colored background showed a distinct shade. When compared on a white ground in diffuse daylight, the specimens in the yellow box showed the lightest shade, those in the red box came next, the specimens in the green box were darker and in blue darkest. No change of color was noticed. All four were

subject to the same light energy from source, but obviously we cannot conclude from this, that quality of light and not brightness of background, determined the effect. These tests of relatively short duration, seemed rather to indicate that brightness and not color or wave-length determined the shade. Further tests were required to settle the point.

In the next series of experiments, the effect of different monochromatic lights on fishes placed in white and black lined boxes was tried. For this purpose Wratten filters were used. These were of the larger size, namely three inches square, the smaller size which were first used not supplying sufficient light surface at the selected distance to illuminate fully the boxes. The filters selected were: Yellow (K, No. 6) Red (F, No. 29) Green (B, No. 68) and Blue (No. 45), approximately corresponding to the four colored backgrounds used in the preceding tests. The Mazda lamps were placed in boxes covered with black paper except the space occupied by the filter set in the middle of the front side. In front of the filter about 60 cm. distant, two boxes were set up, one being lined with dull white and the other with dull black paper, each being so placed as to receive equal amounts of light. All shadows were avoided.

Screened off from this apparatus, a similar one was set up in the same large dark room, so that the effect of each of two monochromatic lights on two different backgrounds could be compared under identical conditions. Each test was of 24-hours duration and comparisons were made on a white ground in diffuse daylight.

When the specimens placed in the white lined boxes were compared, they showed the same sequence in shade as in the previous set of experiments, namely from light to dark; yellow, red, green, and blue, though due to less intensity of light by use of filters, the effect was less pronounced than with the white light on variously colored backgrounds. When the effect of the same monochromatic lights on specimens in the white-lined box was compared to the effect on those in the black-lined box, it was observed that in each case the latter were darker in shade than the former. And moreover little or no difference could be observed in the degree of darkness shown by the specimens in the

black-lined boxes illuminated by different monochromatic lights. The different monochromatic lights have the same effect as white light on a black background.

Parker and Lanchner (1922) suggest the hypothesis that contrast rather than simple vision is involved in responses of this kind. Keeble and Gamble (1904) state that the shade assumed by crustaceans depends on the ratio of light received by the eyes directly from the source, and that received after reflection from the background. Summer (1910) believes that this ratio holds good for fishes. Mast (1916) carried out experiments to test this hypothesis and concluded that while simulation of background is not controlled solely by light reflected from the bottom, the results of the experiments "throw considerable doubt on the hypothesis."

We have made no tests to determine whether the shade depends on this ratio. The experiments merely indicate that the background largly determines the shade. The slight differences in shade of those colored by different monochromatic light is probably due to different amounts of light passing through the filters, and that lights of all colors had a stimulating effect just as white light in causing partial or total contraction of the black pigment cells or melanophores.

From these experiments the conclusion cannot be drawn that it is quality and not quantity of light that determines the shade. The melanophores expand and contract with comparative rapidity in light of different intensities, and as no adaptive coloration was observed in these 24-hour tests the shade assumed was more probably regulated by the different intensities of the various monochromatic lights.

III. Adaptive Changes in Color.

A. Effect of Colored Backgrounds.

In order to test whether *Fundulus* simulates the background in color as some other species had been shown to do, the fishes were placed in battery jars and set in boxes lined with the same colored papers as in the first series of experiments. Here, however, the boxes with the contained fishes were placed with the open side facing the windows of the laboratory and subjected to

the various color stimuli for several weeks. The water was renewed daily, and the temperature never varied more than a few degrees, remaining between about 11 and 15° C. so that the specimens were at all times under the same environmental conditions except that of color. In each jar there were 5 fishes each about 8 cm. in length. On the second days it was noticed that the shade was distinct for the fishes in each of the colored boxes, the sequence from light to darker in shade being yellow, red, green and blue. When these experiments were repeated, it was observed that these differences in shade could be recognized within three or four hours. On the sixth day there was also noticed a distinct color for each group of fishes in the four colored backgrounds. The group in the yellow box were decidedly yellow; those in the red showed a pink color; those in the green a more pronounced green than that sometimes shown by normal specimen, the region above the eyes revealing this tint in a striking manner; while those in the blue box were of a gray, slate-blue color. The adaptation in color increased during the second week when they reached their maximum degree of simulation and maintained it as long as the experiments continued, namely, for about six weeks. This was approximately the maximum time that the specimens could be kept in normal health under the artificial conditions in the laboratory and following this they gradually died. The tests were repeated many times between November 1922 and June 1923 with different sizes of fishes ranging in length from 5 to 10 centimeters and uniform results were obtained. Fundulus simulates the backgrounds after a prolonged stimulation and the effect produced cannot be due to intensity of light alone.

For purposes of comparison, fishes were also placed in three other boxes without color; one lined with dull white paper, a second with neutral gray and a third with dull black paper. Those in the white box showed the pale tint of light-adapted fish; those in the black box a very dark shade, while those in the gray showed an intermediate shade. On the other hand the fishes in the colored boxes each showed a color distinct from the others and from those in the uncolored boxes. The effect, then, cannot be due to intensity of light reflected on the background, but must be due to the color stimuli. Otherwise there is no explanation of the fact

that fishes in the yellow box showed a decidedly yellow color while those in the white box which reflected all light, were pale. Although the melanophores or black pigment cells by their contraction or expansion due to intensity, indirectly influence the color to some extent by exposing or covering other color elements of the skin, the resulting effect of each colored background cannot be explained by this factor. The specific effect is due to color or wave-length.

It was first proved by Pouchet (1876) and later by von Frisch (1911) and other investigators that the expansion of the chromatophores is controlled by the sympathetic nervous system; the eyes act as receptors for the stimuli that cause changes in shade and color. For when the fish were blinded, no adaptive change in shade or color took place. In the experiments on Fundulus, collodion made opaque with lampblack was placed over the eyes and held by stitches of silk thread sewed into the superficial skin. These collodion caps generally remained on for a few days, sufficiently long to show that no adaptive change in shade takes place when fish are blinded, but not long enough to show that adaptive coloration is likewise controlled through the medium of the eye, as has been established by investigators for other species. Other methods were employed but the shock of operation caused changes in color and shade which made the specimens useless for purposes of safe comparison.

Finally it was found that making the cornea opaque with a heated needle had no perceptible influence on the healthy condition of the fishes. Specimens so treated were allowed to remain for a couple of days in order to make sure of their otherwise normal condition before being placed with other fishes in the differently colored backgrounds. In each case the blinded animal showed no simulation of the background.

It is an error to assert, as has been done by some, that blindness necessarily causes maximum darkening of the fish. It depends on the method and length of operation, and different degrees of darkening can be obtained almost at will. The possible objection that making the cornea opaque might still permit the eye to receive the stimulus and thus account for lack of darkening is met by the fact that fishes thus blinded maintain a light shade if placed in

that condition on a black background, which they would not do if the eye were still functioning.

Spaeth (1913) has shown that light, temperature, and also various salts have a direct effect on the contraction of the chromatophores, both melanophores and xanthophores, when the scales with the superficial chromatophores are separated from the fish. But in our experiments with the normal fish, these factors did not influence the results, as the conditions, except background were uniform for all. Sečerov (1909) maintains that the light has a direct action, but this seems disproved by von Frisch.

B. Effect of Spectral Lights or Different Wave-lengths.

The eye being the receptor of the light stimulus, the results of the above experiments can only be explained on the assumption that *Fundulus* objectively discriminates light of different wave-lengths. Since, however, intensity apparently plays an important rôle in the different shades assumed by the fish and to some extent in color, by the relative degree of expansion of the melanophores thus covering or exposing other color elements, a more rigid test was made by using spectral lights of different wave-lengths but of the same intensity.

In much of the work done on color vision in lower animals where the method of food association with definite colors was used, the factor of intensity was either totally disregarded or arrived at only approximately by comparing the relative brightness of the differently colored objects presented. This method of comparison being subjective, can give no exact quantitative data. Filters whether liquid or glass, are likewise unsafe as they are not always monochromatic, nor do they always exclude the infra-red and ultra-violet rays, and of course differ considerably in the amount of light which they allow to pass through. It is therefore necessary that either the brightness of the various spectral lights should be equated by the use of a flicker photometer, as Reeves (1919) has done in food association experiments, or the lights, varying in quality, should be made equal in the quantity of radiant energy. This latter method was first employed by Laurens (1911) at the suggestion of Prof. G. H. Parker.

More recently Laurens and Hooker (1917) have described an

apparatus by means of which twenty-three lights were obtained each thirty wave-lengths in width but equal in radiant energy and extending by steps of ten from 420$\mu\mu$ to 670$\mu\mu$. The Hilger deviation spectrometer was used to obtain spectral lights and the radiant energy equated by means of a thermopile and galvanometer, thus determining the distance of the lamp from the slit of the collimator, in order to give, equal energy for all sets of lights.

This apparatus was used to test the effect of spectral lights on the color of *Fundulus*. It was necessary, however, to select only a few parts of the spectrum. In fact only two sections would be strictly necessary, one of long wave-length the other of short wave-length. Since the spectral lights are of equal energy, the fishes should show the same effect if intensity is the sole factor. If the effect varies in different spectral lights, it can be due only to wave-lengths. Using the data provided by Laurens and Hooker, the following spectral lights with corresponding conditions in the apparatus were selected.

Range of Wave-lengths, in $\mu\mu$.	Position of Drum.	Width of Slit, Mm.	Range of Wave-lengths Either Side of *D* Line.	Distance from Lamp to Slit in Cm.
470–500.....	484.0	3.4	561–622	4.0
520–550.....	534.0	2.3	569.5–622	13.0
560–590.....	574.5	1.8	573.5–606	19.0
620–640.....	625.0	1.3	577–601.5	28.5

For a light-source, a 1000-watt Mazda lamp was used. The lamp was placed in an iron box 12 inches square at the base and 20 inches high. On the front of the box an aperture was made for light. To secure ventilation apertures were made below in front and near the top of the rear side; these were screened by sheets of asbestos. An asbestos funnel was also placed between the aperture of the lamp box and the slit of the collimator. A metal cylinder lined with black paper and closely fitting to the ocular of the telescope conveyed the light to the jar containing the fishes, placed at a distance of 60 cm. The jar, measuring 10x5.5 cm. and 15 cm. high was covered with white paper on all sides except that exposed to the spectral light and screened off from any light leakage from lamp box. The apparatus was set up in a large dark

room, so that no light other than that passing through slit of the spectroscope could enter the jar. As the light band of 30 wave-lengths is quite narrow in the red end of the spectrum, the narrow side of the jar faced the light. Four fishes about 5 cm. long were used in each test. The water was renewed by siphoning and kept at room temperature. Each test ran day and night from Monday to Saturday, when fish were examined.

In the first test, in which the fishes were exposed to the blue light waves between 470 and 500$\mu\mu$ in length, the pronounced blue color observed in specimens subjected to prolonged stimulus of blue background as noted above, was absent, the specimens not differing in any noticeable degree from some normal fish. The second test (green) was likewise inconclusive. This is the most difficult change to verify, for in light adapted fish, a green tint is always present. The third test, that of yellow—560 to 590$\mu\mu$—gave a decided response, all specimens becoming yellow. It likewise gave a basis for judging the preceding tests. For if intensity alone is a factor in the color changes, since it was equal in all the tests, the specimens in the first two should show the same yellow color as in the third. The experiment was repeated with the same fish after they had again assumed the pale tint by being placed on a white background and the response to yellow occurred again in about 36 hours. Repetition, as observed by Mast and others, increases the rapidity of the response. The fourth tests, that of red which included wave-lengths 610 to 640$\mu\mu$ also called forth a yellow response but less pronounced than in the case of yellow rays.

It is worthy of note that in all tests with spectral light of equal intensity, the shade was about the same, the melanophores being contracted. From this it may be concluded that light of any color has a stimulating effect like that of white light in causing contraction of the melanophores. It shows clearly that intensity of light plays an important rôle in the expansion or contraction of the melanophores and thus indirectly affects the colors of fishes by exposing or obscuring other color elements. Nevertheless, the fact that the yellow part of the spectrum caused a decisive change to a yellow tint by expanding the xanthophores or yellow pigment cells, while the intensity remained the same as in other spectral

lights to which the fishes were exposed, is definite proof that in *Fundulus,* the quality or wave-length has a specific effect apart from quantity of light or intensity. That the responses to the various colors is here not so pronounced as in experiments where fishes were placed in colored boxes and exposed to diffuse daylight for several weeks, is due to the fact that the fishes were exposed to spectral lights for only five day periods. Moreover, the area and brightness of the colored environment were much reduced.

IV. The Basis of Adaptive Changes in Shade and Color.

A. The Normal Coloration.

The specimens freshly taken from the sea varied greatly from a yellowish green to black when seen from above. The silvery bars of the adult male with scattered yellow and bronze colored spots are very conspicuous on specimens otherwise dark. The bars are due to the smaller number of melanophores superficial to the scales and to the presence of dense groups of guanophores or iridocytes, which are frequently arranged like branched filaments. The guanophores are highly refractive and when viewed by reflected light, reveal the whole gamut of colors.

As previously stated all specimens were made to assume the pale yellow green tint by being placed for several days in a white dish before being subjected to the various other colored environments. If, now, the living specimens be examined by reflected light under a microscope or with a good hand-lens, it will be seen that there is more than enough material to work on to account for the colorations revealed in the various experiments. The most striking phenomenon is the presence of brilliant points scattered over the dorsal and lateral surfaces. This is especially prominent when direct sunlight is allowed to fall on the fish. The bright points occupy the central portion of many melanophores and in their dark setting appear like jewels. The phenomonon in due to an association of iridescent guanophores or iridocytes with melanophores.

For further detailed study of the color elements, fresh specimens from each of the colored environments were fixed by dipping them in hot water for about 10 seconds, then in cold and

finally examining them in glycerine. When examined under higher magnification, the guanophores are seen to be spindle-shaped bodies having an average length of about 20μ and a maximum breadth of 3 to 4μ. There are other smaller guanophores or iridocytes, ovoid in shape being about 11μ in length and 3 to 4μ in width. These iridocytes are especially found in patches apart from any association with melanophores and give rise to the mottled condition of the male. When associated with melanophores, the guanophores seem to acquire a more brilliant iridescence than when they occur separately. The more common colors reflected in the specimens adapted to a light background are yellow, orange, and green. If the scales are removed, and examined under the microscope, these iridescent points are not to be seen in the melanophores, which remain on the distal portion of the scales after removal. They are located on the proximal portion of the scale and the reflected rays pass through the distal portion of the overlapping scale.

It is well known that many animals show a green coloration though there is no green pigment present. This is true of *Fundulus*. The green tint is due to the combination of the effects of melanophores and xanthophores over a layer which reflects blue or over iridocytes possessing that physical property which Pouchet termed "le cerulescence." When paraffin sections are made through the skin there is to be seen a compact layer of cells occupying the inner part of the dermis next to the muscles. The cells are rectangular in shape with their long axis parallel to the surface. The nuclei are likewise elongate. Cunningham and McMunn (1893) have suggested an ontogenetic relation existing between this layer which they term the argenteum, and the iridocytes.

There is also a close relation in structure and optical properties between the guanophores of the integument and those of the peritoneum. The peritoneum of *Fundulus* is covered with dense black pigment on the surface exposed when the body cavity is opened, and the inner surface shows the typical silvery sheen. This layer is more highly specialized than the reflecting layer of the integument. It does not stain with anilin dyes. If the pigmented surface be examined by deflected light, the same phenomenon of brilliant points associated with melanophores is seen,

showing that the guanophores have the same optical property as was present in the integument.

B. Shade Adapted to White, Gray, and Black Backgrounds.

As is well known for many species, changes in shade are due to contraction or expansion of the melanophores. Ballowitz (1893) has shown that the chromatophores of fishes are innervated by branches which proceed from a dense nerve-net surrounding the chromatophores, or even direct from a nerve bundle itself. These constitute the true pigmento-motor nerves. According to Ballowitz, they have many free terminations on and within the chromatophores. As already pointed out, the eye acts as the receptor and the stimulus is conveyed by way of the sympathetic nervous system to motor fibers innervating the chromatophores as Pouchet surmized and von Frisch clearly demonstrated. Von Frisch (1912) maintains that a centre for contraction of the melanophores exists at the anterior end of the medulla.

On a white background the melanophores and xanthophores are contracted. On a black background the melanophores are expanded and the xanthophores are contracted. In the male specimens the silvery bars and the bright mottled condition of the lateral portions are retained no matter how long the fishes are left in this environment. In fact, they are more pronounced by contrast with the dark ground-color of the fish. No evidence of a reduction or increase in the number of melanophores after a prolonged stimulus in the white or black environment was observed, such as Kuntz (1917) asserts to be the case in *Paralichthys*. On a gray background the specimens were intermediate in shade, the melanophores being partially contracted.

C. Yellow Adapted.

The melanophores in specimens exposed to a yellow background for several weeks were maximally contracted. The xanthophores on the other hand were maximally expanded, to such an extent that they appeared diffused and the limits of the individually pigment cells were difficult to determine. In this condition, the blue reflecting layer is largely concealed. Examined by a reflected light, the brilliant points on the scattered melanophores are very striking, the predominant colors reflected being yellow and orange.

D. Red Adapted.—Vaso-dilation.

The melanophores on the superficial distal portions of the scales were likewise contracted in fishes exposed to a red environment for the same period as in the preceding test, but those in the proximal portions and in deeper parts were not fully contracted. The xanthophores, however, were not fully expanded as in the yellow adapted fish. There was a denser spherical concentration of the pigment giving an orange tint. There are no red pigment cells in *Fundulus*. But the color of the specimens adapted to a red background, was quite distinct from that in the yellow. Instead of the bright yellow of the latter they showed a pink color. It was at first thought that this might be caused by the partially contracted xanthophores giving a deeper yellow or orange to the cells, in combination with the melanophores. On further examination it was seen that the dorsal region of the head, usually pale, was quite red due to the dilated condition of the blood capillaries. The opercular region showed a striking network of vessels. The capillaries of the trunk were likewise dilated giving a pink color to the fish. Before the melanophores are more fully contracted by the prolonged stimulus, the color is brownish.

The pink color is without doubt, due to the blood in the capillaries. As in the cases of experiment with fishes on other colored backgrounds which ran concurrently through the winter months, the results here were always the same. The only exceptions were those fishes taken late in the spring which did not seem normal and were affected by the sudden change in temperature on being taken from the sea. Many of these died and those that lived were exceedingly slow in assuming the light shade when placed in a white vessel before being exposed to the red environment. And strangely enough, though not revealing any blood vessels over trunk and head, the caudal find of one of these specimens was very red, due to vaso-dilation. The general coloration of these late-spring specimens on a red background approached the yellow, but was more sombre. The fact that in these specimens lacking the pink coloration, the blood capillaries were not visible, as in other specimens on a red background, confirms this explanation.

While the distinctive color is then due to the blood in the capillaries, there still remained the possibility that the cutaneous vessels

were equally dilated in fishes adapted to other environments, but that the vessels were covered with melanophores. This seemed to be true of the blue adapted fish, for the dorsal region showed dark lines as if the melanophores were especially abundant and expanded over the capillaries. But the anterior portion of the head, where few melanophores are present, failed to show any dilation. Again, the fishes adapted to a yellow background where melanophores are maximally contracted and likewise those adapted to a white background where both melanophores and xanthophores are contracted, showed no evidence of vaso-dilation.

E. Green Adapted.

In those adapted to the green environment, the shade was darker than in the preceding cases, due to partial expansion of the melanophores. The xanthophores were only slightly expanded. The most striking portion of the body in which this color was emphasized, was above the eyes.

F. Blue Adapted.

In these specimens the melanophores lying in the deeper portions of the integument, and those lying upon the proximal portion of the scales were expanded, thus giving a grayish tone when seen through the overlying adjacent scales. On the distal portion of the scales, the melanophores were generally contracted. The xanthophores were maximally contracted thus exposing the blue reflecting layer. When the surface of the skin was examined by reflected light, the predominant color coming from the brilliant points was a bluish green.

V. Discussion

From the brief outline at the beginning of this paper of the more recent work on the problem of simulation of fishes to the background, it is evident that the majority of investigators have found that the various species tested, do simulate the background and discriminate light of different wave-lengths. To these species, *Fundulus* can unquestionably be added.

Mast (1916) who made a detailed study of two species of flounders and observations on other fishes kept in the aquaria at

Beauford, comes to the general conclusion that "adaptive changes in shade occur in the skin of practically all of the different fishes in the region of Beaufort, N. C.; adaptive changes in color in many, but adaptive changes in pattern in only few."

When the problem of color discrimination is attacked directly by the method of unlearned responses or by forming food associations, the factor of light intensity must be the same for all wave-lengths. Hess, who has done most in the field of color responses in animals generally, stoutly denies that conclusive proof has been brought forth for color vision in fishes, and Parsons (1915) accepts Hess's conclusion.

One benefit of this criticism has been to bring about the use of more precise method in testing the responses of fishes to different colors. Reeves (1919), however, equated brightness for different colors and concluded that fishes discriminate light of different wave-lengths. On the contrary, Ohashi (1921) concludes that intensity alone is responsible for the different responses in the goldfish and carp when subjected to monochromatic light. As this is the most recent paper that has come to our notice, and as the author arrives at conclusions different from those stated in the present paper, we shall discuss Ohashi's work in detail.

Ohashi first noticed that goldfish up to three years old were attracted to different colored lights when subjected in turn to these stimuli. In other words they are positively phototropic to all monochromatic lights.

In the following experiments red and green liquid filters were set in the top of an aquarium equidistant from the ends and the lamp placed midway between them. The fish assembled under the green light which was the brightest for them. When the lamp was adjusted so that the green and red appeared of equal brightness to the observer, the fishes assembled in about equal numbers under each color. From these observations Ohashi concludes that no support for the belief that fishes have color vision can be found here. Apart from the fact that the liquid filters admittedly did not give pure monochromatic lights, there is no certainty that the apparent equality of brightness of the two lights to the human eye were likewise of equal brightness to the eyes of the fish. In short, brightness cannot be objectively determined by

simply comparing the different colored lights and no valid conclusions can be drawn from tests of this kind.

In his food association experiments, Ohashi noted that fishes could not discriminate red when this color was plainly visible to the human eye. This does not disprove color discrimination in fishes for it is quite probable that at a lowered intensity they would be unable to discriminate color and yet be able to do so above a certain threshold of intensity. Hess, in fact, has shown that the red end of the spectrum is shortened for fishes.

When subjected to prolonged stimulation by red and by violet lights the fish became reddish yellow and bluish and on examination it was found that the melanophores expanded in blue light and the xanthophores in yellow light. Unless, therefore, there is a direct effect of light on the chromatophores as Šecerov claimed, but which von Frisch and Mast deny, the reported adaptive coloration must be due to color discrimination.

Ohashi next placed fishes on eight bottoms of colored paper; blue (two tints), red (also two tints) white, black, and gray (two shades). Changes in color were noted in 30 seconds and completed in two minutes, the principle colors noted being dark, reddish yellow and blue. If we have here a case of adaptive coloration, it would indicate a remarkable rapidity in the response. Obviously it is only adaptation in shade, as the author finally concluded when all specimens were compared with one another and it was noted that those which had been on a dark red bottom were similar in shade to those on a grey. The period was entirely too short, however, to conclude from this experiment that they are unable to simulate the background in color.

The same may be said regarding his final experiments where fishes were subjected to the stimulus of red and blue from filters, those in the red becoming reddish yellow and those in the blue, dark within five minutes. When the blue light was made more intense than the red, the fishes in the blue light became yellow and those in the red dark. Again intensity of light was no doubt the factor causing different degrees of contraction of the melanophores as the author concludes and as we have noted for *Fundulus*. This however, does not disprove color discrimination for these species. On the contrary, the results following pro-

longed stimulus of different colored backgrounds indicate that the species tested do simulate the background.

The subjective significance of the phenomonon of color simulation, is not considered here. It is important first to know, as Uhlenhuth (1911) pointed out, whether lights of different wave-length bring about different responses. This has been found to be true of Fundulus heteroclitus.

This research was suggested to me by Prof. G. H. Parker and carried out under his supervision. To Prof. Parker I am indebted for many helpful suggestions, for the privileges of the Harvard Laboratory and for many other courtesies extended while making this investigation.

VI. Summary

1. Adaptive changes in shade occur in *Fundulus heteroclitus* when placed on white, gray, and black backgrounds. This adaptation is brought about by the contraction or expansion of the melanophores.

2. On colored backgrounds for short periods, adaptation in only shade occurs.

3. Prolonged stimulation by colored backgrounds brings about adaptation in color. This occurred in yellow, red, green, and blue environments.

4. The experiments with spectral lights of different wave-lengths but of the same radiant energy or intensity, show that the melanophores respond similarly at all wave-lengths, the contraction being due to light intensity.

5. The response to the longer wave-lengths, red and especially yellow, in causing an expansion of the xanthophores, though intensity was the same as in the blue or short wave-length end of the spectrum, shows that the quality of light or wave-length has a specific influence on the coloration of *Fundulus*.

6. The stimuli causing changes in shade and color are received through the eyes.

7. The changes in color are brought about by the degree of expansion or contraction of the melanophores and xanthophores combined with the optical properties of the guanophores or irido-

cytes and the reflecting layer. The guanophores are frequently found associated with the melanophores.

8. Adaptive change to a yellow background is brought about by a maximum expansion of xanthophores and a maximum contraction of the melanophores.

9. In fishes adapted to a red background, the xanthophores are only partially expanded and the melanophores contracted. The pink color is due chiefly to a dilation of the blood capillaries.

10. In fishes adapted to a green background, the green tints present in the normal light adapted fish, is increased and is especially noticeable above the eyes. The xanthophores and melanophores are partially expanded.

11. In blue adapted fishes the xanthophores are maximally contracted and the melanophores in the deeper portions of the dermis and upon the proximal parts of the scales, are expanded.

Postscript. Since the preparation of this paper I have had the opportunity of repeating the experiments on *Fundulus* at the St. Andrew's Biological Station. My results have been the same as those obtained in the Harvard Laboratory except that I failed to get on a red background the pink coloration and vaso-dilation described in this paper. Whether this is a seasonal or a local difference in the fishes remains to be worked out.

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